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(54) Title: METHOD

(57) Abstract: A method of producing one or more of a carbohydrate ester, a protein ester, a protein subunit ester or a hydroxy acid ester, which method comprises admixing an acyl donor, an acyl acceptor and water to produce a high water environment comprising 5-98% water, wherein said acyl donor is a lipid substrate selected from one or more of the group consisting of a phospholipid, a lysophospholipid, a triacylglyceride, a diglyceride, a glycolipid or a lysoglycolipid and said acyl acceptor is selected from one or more of the group consisting of a carbohydrate, a protein, a protein subunit, or a hydroxy acid; and contacting the admixture with a lipid acyltransferase, such that said lipid acyltransferase catalyses one or both of the following reactions; alcoholysis or transesterification.

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METHOD

REFERENCE TO RELATED APPLICATIONS

Reference is made to the following related applications: United States Application Serial Number 09/750,990 filed on 20 July 1999 and United States Application Serial Number 10/409,391. Each of these applications and each of the documents cited in each of these applications ("application cited documents"), and each document referenced or cited in the application cited documents, either in the text or during the prosecution of those applications, as well as all arguments in support of patentability advanced during such prosecution, are hereby incorporated herein by reference. Various documents are also cited in this text ("herein cited documents"). Each of the herein cited documents, and each document cited or referenced in the herein cited documents, is hereby incorporated herein by reference.

FIELD OF INVENTION

The present invention relates to a method for the bioconversion of lipids to produce a carbohydrate ester and/or a protein ester and/or a protein subunit ester and/or a hydroxy acid ester by use of a lipid acyltransferase.

The present invention further relates to the use of a lipid acyltransferase to bioconvert a lipid into one or more of the following: a carbohydrate ester and/or a protein and/or a protein subunit ester and/or and/or a hydroxy acid ester.

The present invention further relates to the use of an immobilised lipid acyltransferase as defined herein, which immobilised lipid acyltransferase may be used in bioconversion of a lipid in a high water environment to produce one or more of a carbohydrate ester and/or a protein ester and/or a protein subunit ester and/or a hydroxy acid ester.

The present invention yet further relates to an immobilised lipid acyltransferase.

TECHNICAL BACKGROUND

5 Lipases have been extensively used in bioconversion of lipids to make high value products, for example sugar esters, for use in a wide range of industries, including the food and/or feed industries, the cosmetics and/or skin care industries, the oleochemical industry and the pharmaceutical industry.

10 When bioconversion processes require hydrolysis of lipid substrates, lipolytic enzymes can be used in high water environments. However, when bioconversion processes require interesterification or transesterification reactions such as by alcoholysis the use of lipases in high water environments can be detrimental due to unwanted hydrolysis reactions, which result in unwanted bioproducts and/or lower yields of the bioconversion product.

15 Typically, bioconversion processes requiring interesterification and/or transesterification have utilised lipases in non-water environments such as in oil systems and/or in organic solvent systems such as in butanol, methanol or hexane. Such systems provide an environment in which both the polar acceptor molecule and the lipid donor molecule can be at least partially solubilised, and the lipase has
20 sufficient enzyme activity. Although a small amount of water is required for any enzymatic activity, the amount of water is strictly maintained at a low level to avoid hydrolytic activity of the enzyme.

25 Conventionally sugar esters, protein esters or hydroxyacid esters have been produced by chemical synthesis using inorganic catalysts. Convention bioconversion processes for the production of sugar esters or hydroxyacid esters utilise lipases in organic solvent environments or supercritical fluids where there is only a low amount of (if any) water present.

30 Lecointe *et al* Biotechnology Letters, Vol 18., No. 8 (August), pp869-874 disclose a study of a number of lipase enzymes and their activity in an aqueous media on the

production of methyl ester or butyl ester from methanol and butanol, respectively. Lecointe *et al* teach a lipase/acyltransferase from *Candida parapsilosis* which as methanol or butanol concentrations increased showed a reduced hydrolysis activity and an enhanced capability of the enzyme to produce methyl ester and butyl ester. The
5 use of a lipase/acyltransferase from *C. parapsilosis* in the production of fatty hydroxamic acid is taught in Vaysse *et al* J. of Biotechnology 53 (1997) 41-46.

Lipase:cholesterol acyltransferases have been known for some time (see for example Buckley - Biochemistry 1983, 22, 5490-5493). In particular,
10 glycerophospholipid:cholesterol acyl transferases (often referred to as GCATs) have been found, which like the plant and/or mammalian lecithin:cholesterol acyltransferases (LCATs), will catalyse fatty acid transfer between phosphatidylcholine and cholesterol.

15 Upton and Buckley (TIBS 20, May 1995 p 178-179) and Brumlik and Buckley (J. of Bacteriology Apr. 1996 p 2060-2064) teach a lipase/acyltransferase from *Aeromonas hydrophila* which has the ability to carry out acyl transfer to alcohol acceptors in an aqueous media.

20 SUMMARY ASPECTS OF THE PRESENT INVENTION

According to a first aspect of the present invention there is provided a method of producing one or more of a carbohydrate ester, a protein ester, a protein subunit ester or a hydroxy acid ester, which method comprises admixing an acyl donor, an acyl
25 acceptor and water to produce a high water environment comprising 5-98% water, wherein said acyl donor is a lipid substrate selected from one or more of the group consisting of a phospholipid, a lysophospholipid, a triacylglyceride, a diglyceride, a glycolipid or a lysoglycolipid and said acyl acceptor is selected from one or more of the group consisting of a carbohydrate, a protein, a protein subunit or a hydroxy acid;
30 and contacting the admixture with a lipid acyltransferase, such that said lipid acyltransferase catalyses one or both of the following reactions: alcoholysis or transesterification.

In a further aspect the present invention provides use of a lipid acyltransferase to produce one or more of a carbohydrate ester, a protein ester, a protein subunit ester or a hydroxy acid ester by catalysis of one or both of alcoholysis or transesterification in
5 an admixture of an acyl donor, an acyl acceptor and water, which admixture comprises 5-98% water, wherein said acyl donor is a lipid substrate selected from one or more of the group consisting of a phospholipid, a lysophospholipid, a triacylglyceride, a diglyceride, a glycolipid or a lysoglycolipid and said acyl acceptor is selected from one or more of the group consisting of a carbohydrate, a protein, a protein subunit or a
10 hydroxy acid.

In accordance with another aspect of the present invention, there is provided a carbohydrate ester, a protein ester, a protein subunit ester or a hydroxy acid ester produced by a method according to the present invention.
15

In accordance with a further aspect of the present invention, there is provided a pharmaceutical, a cosmetic, a foodstuff, a feedstuff, a paint comprising a carbohydrate ester, a protein ester, a protein subunit ester or a hydroxy acid ester produced by a method according to the present invention.
20

In accordance with a further aspect, the present invention provides an immobilised lipid acyltransferase enzyme as defined herein.

DETAILED ASPECTS OF THE PRESENT INVENTION 25

The term "lipid acyltransferase" as used herein means an enzyme which as well as having lipase activity (generally classified as E.C. 3.1.1.x in accordance with the Enzyme Nomenclature Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology) also has
30 acyltransferase activity (generally classified as E.C. 2.3.1.x), whereby the enzyme is capable of transferring an acyl group from a lipid to one or more of the following acceptor substrates: a carbohydrate; a protein; a protein subunit or a hydroxy acid.

Preferably, the "acyl acceptor" according to the present invention is not water.

5 In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid substrate to a carbohydrate.

The carbohydrate acyl acceptor may be one or more of the following: a monosaccharide, a disaccharide, an oligosaccharide or a polysaccharide. Preferably, the carbohydrate is one or more of the following: glucose, fructose, anhydrofructose,
10 maltose, lactose, sucrose, galactose, xylose, xylooligosacharides, arabinose, maltooligosaccharides, tagatose, microthecin, ascopyrone P, ascopyrone T or cortalcerone.

Carbohydrate esters can function as valuable emulsifiers for example in foodstuffs.
15

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid substrate to a protein and/or a protein subunit.

Preferably the protein sub-unit is one or more of the following: an amino acid, a
20 protein hydrolysate, a peptide, a dipeptide, an oligopeptide, a polypeptide.

Suitable proteins may be one or more of the following: proteins found in a food product, for example in a dairy product and/or a meat product. By way of example only, suitable proteins may be those found in curd or whey, such as lactoglobulin.

25 Other suitable proteins include ovalbumin (from egg), gliadin, glutenin, puroindoline, wheat protein, lipid transfer proteins from grains, myosin from meat, or the following milk proteins: caseins, lactalbumins and lactoferrins.

Suitably in the protein or protein subunit the acyl acceptor may be one or more of the
30 following constituents of the protein or protein subunit: a serine, a threonine, a tyrosine or a cysteine.

When the protein subunit is an amino acid, suitably the amino acid may be any amino acid. Preferably the amino acid is one or more of a serine, a threonine, a tyrosine or a cysteine for example.

- 5 In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid substrate to a hydroxy acid.

Suitably the hydroxy acid may be one or more of the following acids: citric acid, tartaric acid, lactic acid, ascorbic acid, glycolic acid, malic acid, alpha-
10 hydroxyethanoic acid, alpha-hydroxyoctanoic acid, alpha-hydroxycaprylic acid, hydroxycaprylic acid, gluconic acid, lactobionic acid or maltobionic acid.

Suitably the hydroxy acid may be a fruit acid, for example one or more of malic acid, lactic acid, tartaric acid, citric acid or glycolic acid.

15

In one embodiment, preferably the hydroxy acid is one or more of the following acids: citric acid, lactic acid, tartaric acid or malic acid.

The term "hydroxy acid" as used herein means a carboxylic acid in which one or more
20 hydrogen atom of the alkyl group has been replaced by a hydroxyl group.

In one aspect, the lipid acyltransferase may, as well as being able to transfer an acyl group from a lipid substrate to one or more of a carbohydrate, a protein, a protein subunit or a hydroxy acid, the lipid acyltransferase is additionally able to transfer the
25 acyl group from a lipid to one or more of the following: a sterol and/or a stanol, in particular a phytosterol and/or a phytostanol.

Suitably, when the lipid substrate is a phospholipid it may be a lecithin, e.g. phosphatidylcholine. The term lecithin as used herein encompasses
30 phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol.

Suitably, when the lipid substrate is a lysophospholipid it may be a lysolecithin, e.g. lysophosphatidylcholine. The term lysophosphatidylcholine as used herein is synonymous with the term lysolecithin and these terms may be used herein interchangeably.

5

Suitably, when the lipid substrate is a glycolipid it may be digalactosyldiglyceride (DGDG) for example.

The lipid substrate may be referred to herein as the "lipid acyl donor" or "acyl donor".

10 These terms are used interchangeably herein.

For some aspects, preferably the lipid substrate upon which the lipid acyltransferase acts is a phospholipid, such as lecithin, for example phosphatidylcholine.

15 For some aspects, preferably the lipid substrate is a glycolipid, such as DGDG for example.

For some aspects the lipid substrate may be a food lipid, that is to say a lipid component of a foodstuff.

20

For some aspects, the lipid acyltransferase according to the present invention may be incapable, or substantially incapable, of acting on a triglyceride and/or a 1-monoglyceride and/or 2-monoglyceride.

25 Suitably, the lipid substrate or lipid acyl donor may be one or more lipids present in one or more of the following substrates: fats, including lard, tallow and butter fat; oils including oils extracted from or derived from palm oil, sunflower oil, soya bean oil, safflower oil, cotton seed oil, ground nut oil, corn oil, olive oil, peanut oil, coconut oil, and rape seed oil. Lecithin from soya, rape seed or egg yolk is also a suitable lipid
30 substrate. The lipid substrate may be an oat lipid or other plant based material containing galactolipids.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of from 8 to 22 carbons.

For some aspects of the present invention, the lipid may be selected from lipids having
5 a fatty acid chain length of from 16 to 22 carbons, more preferably of from 16 to 20 carbons.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of no greater than 14 carbons, suitably from lipids having a
10 fatty acid chain length of from 4 to 14 carbons, suitably 4 to 10 carbons, suitably 4 to 8 carbons.

Preferably the acyl donor is not a free fatty acid.

15 Preferably, the acyl donor is not a carbohydrate (sugar) ester.

Suitably, the lipid acyltransferase according to the present invention may exhibit one or more of the following lipase activities: glycolipase activity (E.C. 3.1.1.26), triacylglycerol lipase activity (E.C. 3.1.1.3), phospholipase A2 activity (E.C. 3.1.1.4)
20 or phospholipase A1 activity (E.C. 3.1.1.32). The term "glycolipase activity" as used herein encompasses "galactolipase activity".

Suitably, the lipid acyltransferase according to the present invention may have at least one or more of the following activities: glycolipase activity (E.C. 3.1.1.26) and/or
25 phospholipase A1 activity (E.C. 3.1.1.32) and/or phospholipase A2 activity (E.C. 3.1.1.4).

For some aspects, the lipid acyltransferase according to the present invention may have at least glycolipase activity (E.C. 3.1.1.26).

30

Suitably, for some aspects the lipid acyltransferase according to the present invention may be capable of transferring an acyl group from a glycolipid and/or a phospholipid

to one or more of the following acceptor substrates: a carbohydrate, a protein, a protein subunit, a hydroxy acid.

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to a carbohydrate to form at least a carbohydrate ester.

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to a protein or a protein subunit to form at least a protein ester (or a protein fatty acid condensate) or a protein subunit ester.

The term "protein subunit ester" as used herein means the ester formed from any protein subunit, such as a dipeptide ester, an oligopeptide ester, a polypeptide ester or a protein hydrolysate ester for example.

For some aspects, preferably the lipid acyltransferase according to the present invention does not exhibit triacylglycerol lipase activity (E.C. 3.1.1.3).

Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

- (i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to one or more of a carbohydrate, protein, protein subunit or hydroxy acid acyl acceptor to form a new ester, i.e. a carbohydrate ester and/or a protein ester and/or a protein subunit ester and/or a hydroxy acid ester; and
- (ii) the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

Preferably, X of the GDSX motif is L. Thus, preferably the enzyme according to the present invention comprises the amino acid sequence motif GSDL.

5 The GDSX motif is comprised of four conserved amino acids. Preferably, the serine within the motif is a catalytic serine of the lipid acyltransferase enzyme. Suitably, the serine of the GDSX motif may be in a position corresponding to Ser-16 in *Aeromonas hydrophila* lipolytic enzyme taught in Brumlik & Buckley (Journal of Bacteriology Apr. 1996, Vol. 178, No. 7, p 2060-2064).

10 To determine if a protein has the GDSX motif according to the present invention, the sequence is preferably compared with the hidden markov model profiles (HMM profiles) of the pfam database.

15 Pfam is a database of protein domain families. Pfam contains curated multiple sequence alignments for each family as well as profile hidden Markov models (profile HMMs) for identifying these domains in new sequences. An introduction to Pfam can be found in Bateman A *et al.* (2002) Nucleic Acids Res. 30; 276-280. Hidden Markov models are used in a number of databases that aim at classifying proteins, for review see Bateman A and Haft DH (2002) Brief Bioinform 3; 236-245.

20 http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=12230032&dopt=Abstract

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=11752314&dopt=Abstract

25 For a detailed explanation of hidden Markov models and how they are applied in the Pfam database see Durbin R, Eddy S, and Krogh A (1998) Biological sequence analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4. The Hammer software package can be obtained from
30 Washington University, St Louis, USA.

Alternatively, the GDSX motif can be identified using the Hammer software package, the instructions are provided in Durbin R, Eddy S, and Krogh A (1998) Biological sequence analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4 and the references therein, and the HMMER2
5 profile provided within this specification.

The PFAM database can be accessed, for example, through several servers which are currently located at the following websites.

<http://www.sanger.ac.uk/Software/Pfam/index.shtml>

10 <http://pfam.wustl.edu/>

<http://pfam.jouy.inra.fr/>

<http://pfam.cgb.ki.se/>

The database offers a search facility where one can enter a protein sequence. Using the
15 default parameters of the database the protein sequence will then be analysed for the presence of Pfam domains. The GDSX domain is an established domain in the database and as such its presence in any query sequence will be recognised. The database will return the alignment of the Pfam00657 consensus sequence to the query sequence.

20

A multiple alignment, including *Aeromonas salmonicida* or *Aeromonas hydrophila* can be obtained by:

a) manual

25 obtain an alignment of the protein of interest with the Pfam00657 consensus sequence and obtain an alignment of P10480 with the Pfam00657 consensus sequence following the procedure described above;

Or

30 b) through the database

After identification of the Pfam00657 consensus sequence the database offers the option to show an alignment of the query sequence to the seed alignment of

the Pfam00657 consensus sequence. P10480 is part of this seed alignment and is indicated by GCAT_AERHY. Both the query sequence and P10480 will be displayed in the same window.

5 The *Aeromonas hydrophila* reference sequence:

The residues of *Aeromonas hydrophila* GDSX lipase are numbered in the NCBI file P10480, the numbers in this text refer to the numbers given in that file which in the present invention is used to determine specific amino acids residues which, in a preferred embodiment are present in the lipid acyltransferase enzymes of the
10 invention.

The Pfam alignment was performed (Figure 33 and 34):

The following conserved residues can be recognised and in a preferable embodiment
15 may be present in the enzymes for use in the compositions and methods of the invention;

Block 1 - GDSX block

hid hid hid hid Gly Asp Ser hid

20 28 29 30 31 32 33 34 35

Block 2 - GANDY block

hid Gly hid Asn Asp hid

130 131 132 133 134 135

25

Block 3 - HPT block

His

309

30 Where 'hid' means a hydrophobic residue selected from Met, Ile, Leu, Val, Ala, Gly, Cys, His, Lys, Trp, Tyr, Phe.

Preferably the lipid acyltransferase enzyme for use in the compositions/methods of the invention can be aligned using the Pfam00657 consensus sequence.

Preferably, a positive match with the hidden markov model profile (HMM profile) of the pfam00657 domain family indicates the presence of the GDSL or GDSX domain according to the present invention.

5

Preferably when aligned with the Pfam00657 consensus sequence the lipid acyltransferase for use in the compositions/methods of the invention have at least one, preferably more than one, preferably more than two, of the following, a GDSx block, a GANDY block, a HPT block. Suitably, the lipid acyltransferase may have a GDSx block and a GANDY block. Alternatively, the enzyme may have a GDSx block and a HPT block. Preferably the enzyme comprises at least a GDSx block.

10

Preferably, when aligned with the Pfam00657 consensus sequence the enzyme for use in the compositions/methods of the invention have at least one, preferably more than one, preferably more than two, preferably more than three, preferably more than four, preferably more than five, preferably more than six, preferably more than seven, preferably more than eight, preferably more than nine, preferably more than ten, preferably more than eleven, preferably more than twelve, preferably more than thirteen, preferably more than fourteen, of the following amino acid residues when compared to the reference *A.hydrophilia* polypeptide sequence, namely SEQ ID No. 32: 28hid, 29hid, 30hid, 31hid, 32gly, 33Asp, 34Ser, 35hid, 130hid, 131Gly, 132Hid, 133Asn, 134Asp, 135hid, 309His

15

20

The pfam00657 GDSX domain is a unique identifier which distinguishes proteins possessing this domain from other enzymes.

25

The pfam00657 consensus sequence is presented in Figure 1 as SEQ ID No. 1. This is derived from the identification of the pfam family 00657, database version 6, which may also be referred to as pfam00657.6 herein.

30

The consensus sequence may be updated by using further releases of the pfam database.

For example, Figures 33 and 34 show the pfam alignment of family 00657, from database version 11, which may also be referred to as pfam00657.11 herein.

- 5 The presence of the GDSx, GANDY and HPT blocks are found in the pfam family 00657 from both releases of the database. Future releases of the pfam database can be used to identify the pfam family 00657.

10 Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

- (i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to one or more of a carbohydrate, protein, protein subunit or hydroxy acid acyl acceptor to form a new ester, i.e. a carbohydrate ester and/or a protein ester and/or a protein subunit ester and/or a hydroxy acid ester;
- 15 (ii) the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.;
- 20 (iii) the enzyme comprises His-309 or comprises a histidine residue at a position corresponding to His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2 or SEQ ID No. 32).

25 Preferably, the amino acid residue of the GDSX motif is L.

In SEQ ID No. 2 or SEQ ID No. 32 the first 18 amino acid residues form a signal sequence. His-309 of the full length sequence, that is the protein including the signal sequence, equates to His-291 of the mature part of the protein, i.e. the sequence without the signal sequence.

30

Preferably, the lipid acyltransferase enzyme according to the present invention comprises the following catalytic triad: Ser-34, Asp-134 and His-309 or comprises a

serine residue, an aspartic acid residue and a histidine residue, respectively, at positions corresponding to Ser-34, Asp-134 and His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2) or Figure 28 (SEQ ID No. 32). As stated above, in the sequence shown in SEQ ID No. 2 or SEQ ID No. 32 the first 18 amino acid residues form a signal sequence. Ser-34, Asp-134 and His-309 of the full length sequence, that is the protein including the signal sequence, equate to Ser-16, Asp-116 and His-291 of the mature part of the protein, i.e. the sequence without the signal sequence. In the pfam00657 consensus sequence, as given in Figure 1 (SEQ ID No. 1) the active site residues correspond to Ser-7, Asp-157 and His-348.

10

Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

- (i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a first lipid acyl donor is transferred to one or more of a carbohydrate, protein, protein subunit or hydroxy acid acyl acceptor to form a new ester, i.e. a carbohydrate ester and/or a protein ester and/or a protein subunit ester and/or a hydroxy acid ester; and
- (ii) the enzyme comprises at least Gly-32, Asp-33, Ser-34, Asp-134 and His-309 or comprises glycine, aspartic acid, serine, aspartic acid and histidine residues at positions corresponding to Gly-32, Asp-33, Ser-34, Asp-134 and His-309, respectively, in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2) or Figure 28 (SEQ ID No. 32).

25

Suitably, the lipid acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from organisms from one or more of the following genera: *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas* and *Candida*.

30

Suitably, the lipid acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from one or more of the following organisms: *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Streptomyces coelicolor*, *Streptomyces rimosus*, *Mycobacterium*, *Streptococcus pyogenes*, *Lactococcus lactis*,
 5 *Streptococcus pyogenes*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Desulfotobacterium dehalogenans*, *Bacillus sp*, *Campylobacter jejuni*, *Vibrionaceae*, *Xylella fastidiosa*, *Sulfolobus solfataricus*, *Saccharomyces cerevisiae*, *Aspergillus terreus*, *Schizosaccharomyces pombe*, *Listeria innocua*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Mesorhizobium loti*, *Ralstonia solanacearum*, *Xanthomonas*
 10 *campestris*, *Xanthomonas axonopodis* and *Candida parapsilosis*.

In one aspect, preferably the lipid acyltransferase enzyme according to the present invention is obtainable, preferably obtained, from one or more of *Aeromonas hydrophila* or *Aeromonas salmonicida*.

15

Suitably, the lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

- (i) the amino acid sequence shown as SEQ ID No. 2 (see Figure 2)
- (ii) the amino acid sequence shown as SEQ ID No. 3 (see Figure 3)
- 20 (iii) the amino acid sequence shown as SEQ ID No. 4 (see Figure 4)
- (iv) the amino acid sequence shown as SEQ ID No. 5 (see Figure 5)
- (v) the amino acid sequence shown as SEQ ID No. 6 (see Figure 6)
- (vi) the amino acid sequence shown as SEQ ID No. 12 (see Figure 14)
- (vii) the amino acid sequence shown as SEQ ID No. 20 (Figure 16)
- 25 (viii) the amino acid sequence shown as SEQ ID No. 22 (Figure 18)
- (ix) the amino acid sequence shown as SEQ ID No. 24 (Figure 20)
- (x) the amino acid sequence shown as SEQ ID No. 26 (Figure 22)
- (xi) the amino acid sequence shown as SEQ ID No. 28 (Figure 24)
- (xii) the amino acid sequence shown as SEQ ID No. 30 (Figure 26)
- 30 (xiii) the amino acid sequence shown as SEQ ID No. 32 (Figure 28)
- (xiv) the amino acid sequence shown as SEQ ID No. 34 (Figure 30) or

an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, or SEQ ID No. 34.

5

Suitably, the lipid acyltransferase enzyme according to the present invention comprises either the amino acid sequence shown as SEQ ID No. 2 or as SEQ ID No. 3 or SEQ ID No. 32 or SEQ ID No. 34 or comprises an amino acid sequence which has 75% or more, preferably 80% or more, preferably 85% or more, preferably 90% or more, preferably 95% or more, identity with the amino acid sequence shown as SEQ ID No. 2 or the amino acid sequence shown as SEQ ID No. 3 or the amino acid sequence shown as SEQ ID No. 32 or the amino acid sequence shown as SEQ ID No. 34.

10

For the purposes of the present invention, the degree of identity is based on the number of sequence elements which are the same. The degree of identity in accordance with the present invention may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, US53711) (Needleman & Wunsch

15

20

Suitably the lipid acyltransferase enzyme according to the present invention comprises an amino acid sequence which has 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, or SEQ ID No. 34.

25

30

Suitably, the lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

- (a) an amino acid sequence shown as amino acid residues 1-100 of SEQ ID No. 2 or SEQ ID No. 32;
- (b) an amino acid sequence shown as amino acids residues 101-200 of SEQ ID No. 2 or SEQ ID No. 32;
- 5 (c) an amino acid sequence shown as amino acid residues 201-300 of SEQ ID No. 2 or SEQ ID No. 32; or
- (d) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(c) above.

10

Suitably, the lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

- (a) an amino acid sequence shown as amino acid residues 28-39 of SEQ ID No. 2 or SEQ ID No. 32;
- 15 (b) an amino acid sequence shown as amino acids residues 77-88 of SEQ ID No. 2 or SEQ ID No. 32;
- (c) an amino acid sequence shown as amino acid residues 126-136 of SEQ ID No. 2 or SEQ ID No. 32;
- (d) an amino acid sequence shown as amino acid residues 163-175 of SEQ ID No. 2 or
- 20 SEQ ID No. 32;
- (e) an amino acid sequence shown as amino acid residues 304-311 of SEQ ID No. 2 or SEQ ID No. 32; or
- (f) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of
- 25 the amino acid sequences defined in (a)-(e) above.

Suitably, the lipid acyltransferase enzyme according to the present invention may comprise an amino acid sequence produced by the expression or one or more of the following nucleotide sequences:

- 30 (a) the nucleotide sequence shown as SEQ ID No. 7 (see Figure 9);
- (b) the nucleotide sequence shown as SEQ ID No. 8 (see Figure 10);
- (c) the nucleotide sequence shown as SEQ ID No. 9 (see Figure 11);

- (d) the nucleotide sequence shown as SEQ ID No. 10 (see Figure 12);
(e) the nucleotide sequence shown as SEQ ID No. 11 (see Figure 13);
(f) the nucleotide sequence shown as SEQ ID No. 13 (see Figure 15);
(g) the nucleotide sequence shown as SEQ ID No. 21 (see Figure 17);
5 (h) the nucleotide sequence shown as SEQ ID No. 23 (see Figure 19);
(i) the nucleotide sequence shown as SEQ ID No. 25 (see Figure 21);
(j) the nucleotide sequence shown as SEQ ID No. 27 (see Figure 23);
(k) the nucleotide sequence shown as SEQ ID No. 29 (see Figure 25);
(l) the nucleotide sequence shown as SEQ ID No. 31 (see Figure 27);
10 (m) the nucleotide sequence shown as SEQ ID No. 33 (see Figure 29);
(n) the nucleotide sequence shown as SEQ ID No. 35 (see Figure 31);
(o) or
a nucleotide sequence which has 75% or more identity with any one of the sequences
shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No.
15 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No.
27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33 or SEQ ID No. 35.

Suitably the nucleotide sequence may have 80% or more, preferably 85% or more,
more preferably 90% or more and even more preferably 95% or more identity with any
20 one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID
No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No.
25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33 or SEQ ID No.
35.

- 25 In one aspect, the lipid acyltransferase according to the present invention may be a
lecithin:cholesterol acyltransferases (LCAT) or variant thereof (for example a variant
made by molecular evolution)

Suitable LCATs are known in the art and may be obtainable from one or more of the
30 following organisms for example: mammals, rat, mice, chickens, *Drosophila*
melanogaster, plants, including *Arabidopsis* and *Oryza sativa*, nematodes, fungi and
yeast.

In one embodiment the lipid acyltransferase enzyme according to the present invention may be the lipid acyltransferase obtainable, preferably obtained, from the *E. coli* strains TOP 10 harbouring pPet12aAhydro and pPet12aASalmo deposited by Danisco
5 A/S of Langebrogade 1, DK-1001 Copenhagen K, Denmark under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure at the National Collection of Industrial, Marine and Food Bacteria (NCIMB) 23 St. Machar Street, Aberdeen Scotland, GB on 22 December 2003 under accession numbers NCIMB 41204 and NCIMB 41205, respectively.

10

The term "transferase" as used herein is interchangeable with the term "lipid acyltransferase".

Suitably, the lipid acyltransferase as defined herein catalyses one or both of the
15 following reactions: transesterification, alcoholysis.

Thus in accordance with the present invention, one or more of the following advantageous properties can be achieved: the bioconversion of lipids to form one or more of a carbohydrate ester, a protein ester, a protein subunit ester or a hydroxy acid
20 ester can take place in a high water environment which comprises no organic solvent or a reduced amount of organic solvent compared with conventional bioconversion processes.

The term "bioconversion" as used herein means the modification of one organic
25 compound to produce another organic compound and/or synthesis of organic compounds from other organic compounds by enzyme catalysis.

The term "transesterification" as used herein means the enzymatic catalysed transfer of an acyl group from a lipid donor (other than a free fatty acid) to an acyl acceptor (other
30 than water). For the avoidance of doubt, the use of the term "transesterification" as used herein includes transfer of an acyl group from a lipid donor to an acyl acceptor

(other than water) where the acyl acceptor comprises a suitable chemical group, which may for example be either an -OH or -SH group.

5 As used herein, the term "alcoholysis" refers to the enzymatic cleavage of a covalent bond of an acid derivative by reaction with an alcohol group ROH so that one of the products combines with the H of the alcohol group and the other product combines with the OR group of the alcohol group.

10 As used herein, the term "hydrolysis" refers to the enzymatic catalysed transfer of an acyl group from a lipid to the OH group of a water molecule. Acyl transfer which results from hydrolysis requires the separation of the water molecule.

15 The term "interesterification" refers to the enzymatic catalysed transfer of acyl groups between a lipid donor and lipid acceptor, wherein the lipid donor is not a free acyl group. In other words "interesterification" refers to the interchange of a fatty acid between two lipid molecules.

In one aspect, the lipid acyl transferase as defined herein catalyses interesterification.

20 Suitably, the method or use according to the present invention may further comprise one or more of the following steps: dissolving the acyl acceptor in water; adding a lipid acyl donor to a dissolved acyl acceptor to form a two-phase system or an emulsion; stirring or sonicating the reaction mixture; heating the reaction mixture, for example to denature the enzyme; separating the water phase from the fat/emulsifier
25 phase by standard separation techniques, such as solvent extraction or water evaporation for example; fractionating the fat phase by hydrophobic interaction chromatography, crystallisation or high vacuum distillation. Suitably, one or more of the heating, separating or fractionating steps may be carried out after the reaction has reached equilibrium.

30

In one embodiment the lipase acyl transferase for use in the methods of the present invention may be immobilised. When it is the case that the enzyme is immobilised the

admixture comprising an acyl donor, an acyl acceptor and water passed through a column for example comprising the immobilised enzyme. By immobilising the enzyme it is possible to easily reuse it.

- 5 Suitably the immobilised enzyme may be used in a flow reactor or in a batch reactor containing a reaction mixture which comprises an acyl acceptor dissolved in water and a lipid acyl donor as a two-phase system or as an emulsion. The reaction mixture may be optionally stirred or sonicated. Once the reaction has reached equilibrium for example, the reaction mixture and the immobilised enzyme may be separated.
- 10 Suitably, the reaction product may be fractionated for example by hydrophobic interaction chromatography, crystallisation or high vacuum distillation.

Immobilised lipid acyl transferase can be prepared using immobilisation techniques known in the art. There are numerous methods of preparing immobilised enzymes,

15 which will be apparent to a person skilled in the art (for example the techniques referred to in EP 0 746 608; or Balcao V.M., Paiva A.L., Malcata F.X., *Enzyme Microb Technol.* 1996 May 1;18(6):392-416; or Retz M.T., Jaeger K.E. *Chem Phys Lipids.* 1998 Jun;93(1-2):3-14; Bornscheuer U.T., Bessler C, Srinivas R, Krishna S.H. *Trends Biotechnol.* 2002 Oct; 20(10):433-7; Plou et al, *J. Biotechnology* 92 (2002) 55-

20 66; Warmuth et al., 1992. *Bio Forum* 9, 282-283; Ferrer et al., 2000. *J. Chem. Technol. Biotechnol.* 75, 1-8; or Christensen et al., 1998. *Nachwachsende Rohstoff* 10, 98-105; Petersen and Christenen, 2000, *Applied Biocatalysis*. Harwood Academic Publishers, Amsterdam. (each of which is incorporated herein by reference). Techniques which may be used herein include covalent coupling to Eupergit C, adsorption on

25 polypropylene and silica-granulation for example.

The term "high water environment" as used herein preferably means an environment which is low in or absent an organic solvent, preferably low in or absent a polar organic solvent. The term organic solvent as used herein preferably does not

30 encompass food oils when used as lipid substrate, and preferably does not encompass food oils that are high in non-polar lipids for example. Suitably, the high water environment according to the present invention may comprise less than 50% by

volume organic solvents, less than 30% by volume organic solvents, more preferably less than 15% by volume organic solvents, more preferably less than 5%, more preferably less than 1%, more preferably less than 0.5% by volume organic solvent, more preferably 0% by volume organic solvents.

5

When it is the case that a carbohydrate ester is produced in accordance with the present invention, the carbohydrate ester is preferably an oligosaccharide ester, a monosaccharide ester or a disaccharide ester.

10 Suitably, the carbohydrate ester when produced in accordance with the present invention may be one or more of the following: glucose ester, fructose ester, anhydrofructose ester, maltose ester, lactose ester, galactose ester, xylose ester, xylooligosaccharide ester, arabinose ester, maltooligosaccharide ester, tagatose ester, sucrose ester, microthecin ester, ascopyrone P ester, ascopyrone T ester or cortalcerone
15 ester.

Preferably, the carbohydrate ester when produced in accordance with the present invention is one or more of the following: a carbohydrate mono-ester, a sugar mono-ester, an oligosaccharide mono-ester, a trisaccharide mono-ester, a disaccharide mono-
20 ester, a monosaccharide mono-ester, a glucose mono-ester, a fructose mono-ester, anhydrofructose mono-ester, maltose mono-ester, lactose mono-ester, galactose mono-ester, xylose mono-ester, xylooligosacchride mono-ester, arabinose mono-ester, maltooligosaccharide mono-ester, tagatose mono-ester, sucrose mono-ester, microthecin ester, ascopyrone P ester, ascopyrone T ester or cortalcerone ester.

25

In one embodiment, the microthecin ester, ascopyrone P ester, ascopyrone T ester and/or cortalcerone ester may function as an antimicrobial agent. Alternatively or in addition thereto, the microthecin ester, ascopyrone P ester, ascopyrone T ester and/or cortalcerone ester may function as one or both of an antioxidant and/or emulsifier.

30

Preferably, the formation of the carbohydrate ester (if any) in accordance with the present invention is independent of UDP-glucose.

Preferably, the foodstuff according to the present invention does not comprise UDP-glucose, or only comprises UDP-glucose in insignificant amounts.

- 5 The lipid acyl transferases used in the compositions and methods of the invention have been found to have unique properties when compared to lipolytic enzymes in that they have a marked preference for transfer of acyl groups from lipids to acceptors other than water, even in the presence of significant water. In a comparison with prior art enzymes, the lipid acyl transferase used in the invention were found to have a high
10 relative transferase activity in the presence of 6% water, 54% water, 73% water, 89% water and approximately 95%. Lipolytic enzymes tested had virtually no significant relative transferase activity at these water concentrations.

The % transferase activity (i.e. the transferase activity as a percentage of the total
15 enzymatic activity) may be determined by the following protocol:

Protocol for the determination of % acyltransferase activity:

A substrate to which a lipid acyltransferase according to the present invention has been
20 added may be extracted following the enzymatic reaction with $\text{CHCl}_3:\text{CH}_3\text{OH}$ 2:1 and the organic phase containing the lipid material is isolated and analysed by GLC and HPLC according to the procedure detailed hereinbelow. From the GLC and HPLC analyses the amount of free fatty acids and one or more of carbohydrate esters, protein esters; protein subunit esters; hydroxy acid esters are determined. A control substrate
25 to which no enzyme according to the present invention has been added, is analysed in the same way.

Calculation:

From the results of the GLC and HPLC analyses the increase in free fatty acids and
30 carbohydrate esters and/or protein esters and/or protein subunit esters and/or hydroxy acid can be calculated:

Δ % fatty acid = % Fatty acid(enzyme) - % fatty acid(control); Mv fatty acid = average molecular weight of the fatty acids;

A = Δ % protein ester/Mv protein ester (where Δ % protein ester = % protein ester(enzyme) - % protein ester(control) and Mv protein ester = average molecular weight of the protein esters) – applicable where the acyl acceptor is a protein;

B = Δ % carbohydrate ester/Mv carbohydrate ester (where Δ % carbohydrate ester = % carbohydrate ester(enzyme) - % carbohydrate ester(control) and Mv carbohydrate ester = average molecular weight of the carbohydrate ester) - applicable where the acyl acceptor is a carbohydrate;

C = Δ % protein subunit ester/Mv protein subunit ester (where Δ % protein subunit ester = % protein subunit ester(enzyme) - % protein subunit ester(control) and Mv protein subunit ester = average molecular weight of the protein subunit ester) – applicable where the acyl acceptor is a protein subunit; and

D = Δ % hydroxy acid ester/Mv hydroxy acid ester (where Δ % hydroxy acid ester = % hydroxy acid ester(enzyme) - % hydroxy acid ester(control) and Mv hydroxy acid ester = average molecular weight of the hydroxy acid ester) – applicable where the acyl acceptor is a hydroxy acid.

The transferase activity is calculated as a percentage of the total enzymatic activity:

$$\% \text{ transferase activity} = \frac{A^* + B^* + C^* + D^* \times 100}{A^* + B^* + C^* + D^* + \Delta \% \text{ fatty acid} / (\text{Mv fatty acid})}$$

* - delete as appropriate.

The lipase and acyltransferase activity of an enzyme may be evaluated using the following assays. In this way, a lipid acyltransferase having the enzyme characteristics defined herein may be obtained/identified.

Transferase Assay in Buffered Substrate (see Example 6)

Enzymes which function as lipid acyltransferases for use in the compositions and methods of the invention can be routinely identified using the assay taught herein in

Example 6. This assay will be hereinafter referred to as the 'Transferase Assay in Buffered Substrate'. In Example 6 the lipid acyltransferase enzyme from *Aeromonas salmonicida* in accordance with the present invention was analysed and compared with a range of lipolytic enzymes not encompassed by the present invention. As can be
5 seen, of the lipolytic enzymes only LIPOPAN® F (Novozymes, Denmark) was found to have any transferase activity and then only a very low level (1.3%).

Enzymes suitable for use in the compositions and methods of the invention can be routinely identified using the Transferase Assay in Buffered Substrate. Using this
10 assay, in which there is a very high water content – approximately 95%, lipid acyltransferases in accordance with the present invention are those which have at least 2% acyltransferase activity (relative transferase activity), preferably at least 5% relative transferase activity, preferably at least 10% relative transferase activity, preferably at least 15%, 20%, 25% 26%, 28%, 30%, 40% 50%, 60% or 75% relative
15 transferase activity. Suitably, the lipid acyltransferase in accordance with the present invention may have less than 28%, less than 30%, preferably less than 40%, 50%, 60%, 70%, 80%, 90% or 100% acyltransferase activity.

Transferase Assay in a Low Water Environment

20

As an alternative to (or in addition to) using the "Transferase Assay in Buffered Substrate", lipid acyltransferases for use in accordance with the present invention may be identified using the "Transferase Assay in a Low Water Environment".

25 In order to determine if an enzyme is a lipid acyltransferase according to the present invention, one may carry out a "Transferase Assay in a Low Water Environment", namely in an oily environment with 6% water as taught in Example 9. This example illustrates that in an oily environment with 6% water content the lipid acyltransferase of the invention has a high relative transferase activity, where the prior art lipolytic
30 enzymes have hydrolytic activity.

In one embodiment, the lipid acyltransferase suitable for use in the methods and/or uses according to the present invention is one which when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%,
5 preferably at least 2%, preferably at least 5%, preferably at least 10%, preferably at least 20%, preferably at least 30%, preferably at least 40%, preferably at least 50%, preferably at least 60%, preferably at least 70%, preferably at least 75%. Suitably, the lipid acyl transferase in accordance with the present invention may have less than 30%, 40%, 50%, 60%, 70%, or 80% activity when measured after a time period of 10,
10 20, 30 or 120 minutes using the "Transferase Assay in a Low Water Environment".

As described above, the lipase acyltransferase of the invention can be identified using either the "Transferase Assay in Buffered Substrate" or in the "Transferase Assay in Low Water Environment" using cholesterol as the acyl acceptor. Of course, the
15 skilled person would be readily aware that, with obvious amendments to the analytical methods the 'Transferase Assay in Buffered Substrate' or the 'Transferase Assay in Low Water Environment' may be used to determine the lipid acyltransferase activity for any lipid acyl donor or any acyl acceptor combination. The skilled person would, if necessary, simply replace the acyl donor substrate (e.g. phospholipid) with an
20 alternative acyl donor substrate (e.g. glycolipid, triacylglyceride) and/or replace the acyl acceptor (e.g. cholesterol) with an alternative acyl acceptor substrate (e.g. a carbohydrate, a protein, a protein subunit or a hydroxy acid) (for example see Examples 10-13).

25 The term "high water environment" as used herein means any environment comprising 5-98% water. Preferably the environment comprises more than 6% water content, preferably more than 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%. Suitably, the high water environment may be comprised of 20-98%, suitably 50-98%, suitably of 70-98%, suitably 75-98% water.

In one embodiment, in the admixture the ratio of the amount of lipid acyltransferase added compared with water is at least 1:700, preferably 1:10,000, as measured on a by weight basis.

- 5 The term "low water" as used herein means any substrate or foodstuff with less than 5% water content, preferably less than 4%, 3%, 2%, 1% or 0.5%.

Preferably the method and/or use according to the present invention may be carried out at a temperature of 15-60°C, preferably at a temperature of 20-60°C, preferably 20-
10 50°C, preferably 20-45°C, preferably 20-40°C.

Suitably, the method or use according to the present invention comprises a further step or purifying and/or isolating the reaction product, namely one or more of a carbohydrate ester a protein ester, a protein subunit ester, or a hydroxy acid ester.
15 Thus, preferably the reaction product is in a purified and/or isolated form.

Numerous methods for purification of esters are known to the skilled person. By way of example only the esters produced by the methods/uses taught herein may be purified using chromatography, such as hydrophobic interaction, filtration, centrifugation,
20 solvent extraction/distillation or crystallisation. Suitable methodologies are taught in Ulmann's Encyclopedia of Industrial Chemistry (2002) by Wiley-VCH Verlag GmbH & Co. KGaA.

The lipid acyl-transferase of the invention may be expressed in any suitable expression
25 host. For example the lipid acyltransferase of the invention may be expressed in *Bacillus subtilis* and may be purified by ultrafiltration and/or by precipitation in ethanol and/or centrifugation, and may be subsequently spray dried using starch (maltodextrin) as carrier for the enzyme. The spray-dried enzyme may be standardized to specified PLU activity by adding further carrier in powder form. The techniques
30 involved are well established and routine in the art.

In one embodiment, the method according to the present invention is an *in vitro* process. The method may suitably be a continuous or batch process.

The enzyme according to the present invention may be used in combination with one or more other further enzymes. Thus, it is within the scope of the present invention that, in addition to the enzyme of the invention, the admixture is contacted with at least one further enzyme. Such further enzymes include starch degrading enzymes such as endo- or exoamylases, pullulanases, debranching enzymes, hemicellulases including xylanases, cellulases, oxidoreductases, e.g. glucose oxidase or a carbohydrate oxidase such as one which oxidises maltose, for example hexose oxidase (HOX), lipases, phospholipases and hexose oxidase, and proteases. The admixture may be contacted with the enzyme of the invention and the at least one further enzyme at the same time or sequentially.

In one embodiment for example the lipid acyltransferase may be used in combination with a lipase having one or more of the following lipase activities: glycolipase activity (E.C. 3.1.1.26, triacylglycerol lipase activity (E.C. 3.1.1.3), phospholipase A2 activity (E.C. 3.1.1.4) or phospholipase A1 activity (E.C. 3.1.1.32). Suitable lipase enzymes are well known within the art and include by way of example the following lipases: LIOPAN[®] F and/or LECITASE[®] ULTRA (Novozymes A/S, Denmark), phospholipase A2 (e.g. phospholipase A2 from LIPOMOD[™] 22L from Biocatalysts, LIPOMAX[™] from Genecor), LIPOLASE[®] (Novozymes A/S, Denmark), the lipases taught in WO03/97835, EP 0 977 869 or EP 1 193 314.

25 USES

Thus, the methods according to the present invention produce one or more of a carbohydrate ester, a protein ester, a protein subunit ester, a hydroxyacid ester. Many of these esters are useful emulsifiers. By way of example only amino acid esters, peptide esters, protein esters, carbohydrate esters and hydroxy acid esters (such as tartaric acid esters) for example are functionally important emulsifiers. Emulsifiers are useful in a wide range of industries, such as the food industry, the feed industry, the

cosmetics industry (for example in cosmetic bases), the pharmaceutical industry (in both pharmaceutical synthesis and formulation for example) and the paint industry for example. Emulsifiers can function as wetting agents, food ingredients and active ingredients.

5

In addition protein fatty acid condensates owing to their excellent physiological properties, are suited for use in cosmetics and personal hygiene products for example. For example, protein esters may be used in shower and bath preparations as well as in shampoos and body cleansers. The protein fatty acid condensates may also be useful
10 in pharmaceutical compositions, for example as a base.

Protein fatty acid condensates are well known for their application in the cosmetic industry. Conventionally, these products are produced by reacting protein hydrolyzate with fatty acid chloride under Schotten-Baumann conditions, using water as solvent.
15 (http://www.scf-online.com/english/26_e/rawmaterials26_e.htm#5).

In the development of the protein-fatty acid condensates it is possible to combine the renewable resources fatty acids (from vegetable oil) and protein, which can be obtained from both animal waste (leather) as well as from many plants, to construct a
20 surfactant structure with a hydrophobic (fatty acid) and a hydrophilic (protein) part. In this process the fatty acid chloride reacts with the amine group of the amino acid and forms the protein fatty acid condensate (See Figure 49). Products are obtained which have an excellent skin compatibility and additionally have a good cleaning effect.

25 The fact that even small additions of the acylated protein hydrolysate have a synergistic effect on the skin compatibility of other surfactants is highly important from a technical formulation point of view. An explanation for this protective effect could lie in the amphoteric behaviour of the product. There is an interaction between the protein-fatty acid condensate and skin collagen. This leads to the formation of a
30 protective layer, which reduces the excessive attack of surfactants on the upper layers of the skin, their strong degreasing effect and the direct interaction of anionic surfactants with the skin.

In the cosmetic branch, protein-based surfactants are mainly used in mild shower and bath products, mild shampoos, surfactant-based face cleansers, cold-wave preparations and fixatives or surfactant preparations for babies.

5

Protein hydrolysate fatty acid condensates are also useful as bases for pharmaceutical preparations, for example for creams and ointments which contain active ingredients for topical application to the skin.

- 10 The present invention provides a new way to produce protein fatty acid condensate without using fatty acid chloride. The reaction according to the present invention is depicted in Figure 50. This reaction can be conducted in water or buffer system at low temperature without formation of waste products.

- 15 The term "protein fatty acid condensate" as used herein encompasses all of the following protein esters, polypeptide esters, dipeptide esters, oligopeptide esters, peptide esters, and amino acid esters.

- As a skilled person would be readily aware, carbohydrate esters (particularly sugar
20 esters) have a broad application in the food industry. Other fields of application include cosmetics, oral-care products and medical supplies. In addition, these compounds can be used as antibiotics, antitumorals, fungicides and insecticides. The lipid acyltransferase according to the present invention is able to catalyse the formation of glucose ester in a high water environment (Figure 51).

25

The esters produced in accordance with the present invention find application in the following fields:

- Cosmetics: including essential oil emulsions (o/w, HLB 16-18) Paraffin oil emulsions,
30 o/w, HLB 10 – 14; Stearic acid emulsions; Wax emulsions, o/w, HLB 14 – 16; Lanolin emulsions, o/w, HLB 12 – 14; Silicone emulsions; Toothpastes, o/w; Foam baths, o/w, HLB 14 – 18; Hair Lotion.

Pharmaceutical Preparations: including in drug emulsions; ointment bases; suppository compound, w/o; encapsulation; injection preparation.

- 5 Agriculture: including in soil improvement; as a fertiliser additive; as all-purpose cleaners; cleaners for fruit and vegetables; cleaners for milk churns.

Crop Protection: including in naturally occurring insecticides; chlorinated hydrocarbons, and 140; phosphoric acid esters o/w, HLB 10 – 14; fungicides, o/w;
10 herbicides, o/w.

Food Industry: including in bread and cakes; margarine; chocolate; fat bloom prevention, w/o, HLB 5 – 10; sugar frosting, o/w, HLB 14 – 16; softeners for caramels and chewing gum, w/o, HLB. 2 – 4; prevention of sticking, w/o, HLB 2 – 4;
15 ice cream additives w/o, HLB 4 – 6; wetting of milk and baking powders, w/o, HLB 9 – 11; custard powder, w/o, HLB 2 – 4; in the drinks industry; in fruit and vegetables; in flavourings, w/o and o/w, HLB 10 – 12; in meat, salad, or other flavouring sauces, o/w; in food dyes, w/o, HLB 2 – 4; o/w, HLB 8 – 18; in foam inhibitors.

20

The benefit of using protein fatty acid esters, hydroxy acid esters and carbohydrate esters produced in accordance with the present invention as emulsifiers in food applications is that these are harmless food compatible components which are more easily biodegradable compared to other conventionally used emulsifier like
25 ethoxylated fatty acid esters for example. These emulsifiers are thus more environmentally friendly to use in both the food industry and the non-food industry.

In one embodiment, the microthecin ester, ascopyrone P ester, ascopyrone T ester and/or cortalcerone ester may function as an antimicrobial agent. Alternatively or in
30 addition thereto, the microthecin ester, ascopyrone P ester, ascopyrone T ester and/or cortalcerone ester may function as one or both of an antioxidant and/or emulsifier

In one embodiment, the methods or uses of the present invention can be used to produce emulsifiers for use in drug formulations, particularly in the production of controlled release formulations of active ingredients, wherein the active ingredient is acylated using the lipid acyl-transferase. Such slow release formulations are particularly useful for pharmaceutical compositions administered orally, where the gradual hydrolysis of the ester in the digestive tract provides gradual delivery of the active ingredient. Such acylated compositions could further be used for a subcutaneous or an intravenous formulation.

10 In another embodiment, the methods or uses of the present invention can be used to produce phase transfer catalysts for transfer of salts into a solution of organic solvents for instance in an organic reaction. For example, the transfer of an acyl group to an appropriate cationic acceptor, such as a hydroxy acid (citric acid), or alternatively with an anionic acceptor group, such as hydroxy-amines can produce phase transfer catalysts for transfer of salts into a solution of organic solvents.

In another embodiment, the methods of the present invention may be used to produce ester prodrugs of pharmaceutical compounds with low biological availability and/or low solubility, for instance antiviral agents like aciclovir and gangaciclovir. The method could further be used for other medicinal compounds with a free hydroxy-group, for instance a primary, secondary or tertiary hydroxy-group.

25 Preferably, the ester produced in accordance with the present invention is used in a pharmaceutical formulation.

Preferably, the ester produced in accordance with the present invention is used in a cosmetic and/or a personal hygiene product.

30 Preferably, the ester produced in accordance with the present invention is used in a foodstuff and/or a feedstuff.

The method in accordance with the present invention may be one step in the manufacturing process of one or more of a pharmaceutical, a cosmetic, a personal hygiene product a foodstuff or a feedstuff.

5 ADVANTAGES

One advantage of the method according to the present invention is that it results in the manufacture of one or more of a carbohydrate ester, a protein ester, a protein subunit ester or a hydroxy acid ester without the need to use organic solvents. Thus, the
10 present invention allows the use of the organic solvents to be reduced or eliminated. This has many advantages, for example in reduced production costs, reduced human and/or environmental exposure to organic solvents, simplification of the production process.

15 In the production of esters for food applications it is particularly advantageous to use lipids rather than fatty acids because it is not necessary to remove surplus lipids because these can form part of the food item where the reaction product is used. On the other hand, surplus free fatty acids would have to be removed because these are deleterious for most food products.

20

ISOLATED

In one aspect, preferably the polypeptide or protein for use in the present invention is in an isolated form. The term "isolated" means that the sequence is at least
25 substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature.

In one aspect, preferably the bioconversion product according to the present invention for example the carbohydrate ester and/or the protein ester and/or the protein subunit
30 ester and/or the hydroxy acid ester is isolated from the reaction mixture. The term "isolated" means that the bioconversion product is at least substantially free from at

least one other component with which the bioconversion product is associated during the bioconversion reaction.

PURIFIED

5

In one aspect, preferably the polypeptide or protein for use in the present invention is in a purified form. The term "purified" means that the sequence is in a relatively pure state – e.g. at least about 51% pure, or at least about 75%, or at least about 80%, or at least about 90% pure, or at least about 95% pure or at least about 98% pure.

10

In one aspect, preferably the bioconversion product produced in accordance with the present invention, for example the carbohydrate ester and/or the protein ester and/or the protein subunit ester and/or the hydroxy acid ester is purified from the reaction mixture and is therefore in a purified form. The term "purified" means that the bioconversion product is in a relatively pure state – e.g. at least about 51% pure, or at least about 75%, or at least about 80%, or at least about 90% pure, or at least about 95% pure or at least about 98% pure.

15

PHARMACEUTICAL COMPOSITIONS

20

The present invention also provides a pharmaceutical composition comprising the product of the present invention and a pharmaceutically acceptable carrier, diluent or excipient (including combinations thereof).

25

The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier,

30

excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be administered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestible solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be administered by a number of routes.

Where the agent is to be administered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit through the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual

administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

CLONING A NUCLEOTIDE SEQUENCE ENCODING A POLYPEPTIDE 5 ACCORDING TO THE PRESENT INVENTION

A nucleotide sequence encoding either a polypeptide which has the specific properties as defined herein or a polypeptide which is suitable for modification may be isolated from any cell or organism producing said polypeptide. Various methods are well
10 known within the art for the isolation of nucleotide sequences.

For example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the polypeptide. If the amino acid sequence of the polypeptide is known, labelled oligonucleotide
15 probes may be synthesised and used to identify polypeptide-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known polypeptide gene could be used to identify polypeptide-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.

20 Alternatively, polypeptide-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing an enzyme inhibited by the polypeptide,
25 thereby allowing clones expressing the polypeptide to be identified.

In a yet further alternative, the nucleotide sequence encoding the polypeptide may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beucage S.L. *et al* (1981) Tetrahedron Letters 22, p 1859-1869,
30 or the method described by Matthes *et al* (1984) EMBO J. 3, p 801-805. In the

phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

5 The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in
10 Saiki R K *et al* (Science (1988) 239, pp 487-491).

NUCLEOTIDE SEQUENCES

15 The present invention also encompasses nucleotide sequences encoding polypeptides having the specific properties as defined herein. The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-
20 stranded or single-stranded whether representing the sense or antisense strand.

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence.

25

In a preferred embodiment, the nucleotide sequence *per se* encoding a polypeptide having the specific properties as defined herein does not cover the native nucleotide sequence in its natural environment when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred
30 embodiment the "non-native nucleotide sequence". In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally

associated, which promoter is also in its native environment. Thus, the polypeptide of the present invention can be expressed by a nucleotide sequence in its native organism but wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

5

Preferably the polypeptide is not a native polypeptide. In this regard, the term "native polypeptide" means an entire polypeptide that is in its native environment and when it has been expressed by its native nucleotide sequence.

- 10 Typically, the nucleotide sequence encoding polypeptides having the specific properties as defined herein is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative embodiment of the invention, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23
15 and Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

MOLECULAR EVOLUTION

- Once an enzyme-encoding nucleotide sequence has been isolated, or a putative
20 enzyme-encoding nucleotide sequence has been identified, it may be desirable to modify the selected nucleotide sequence, for example it may be desirable to mutate the sequence in order to prepare an enzyme in accordance with the present invention.

- Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides
25 contain nucleotide sequences flanking the desired mutation sites.

A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151).

30

Instead of site directed mutagenesis, such as described above, one can introduce mutations randomly for instance using a commercial kit such as the GeneMorph PCR

mutagenesis kit from Stratagene, or the Diversify PCR random mutagenesis kit from Clontech. EP 0 583 265 refers to methods of optimising PCR based mutagenesis, which can also be combined with the use of mutagenic DNA analogues such as those described in EP 0 866 796. Error prone PCR technologies are suitable for the
5 production of variants of lipid acyl transferases with preferred characteristics. WO0206457 refers to molecular evolution of lipases.

A third method to obtain novel sequences is to fragment non-identical nucleotide sequences, either by using any number of restriction enzymes or an enzyme such as
10 Dnase I, and reassembling full nucleotide sequences coding for functional proteins. Alternatively one can use one or multiple non-identical nucleotide sequences and introduce mutations during the reassembly of the full nucleotide sequence. DNA shuffling and family shuffling technologies are suitable for the production of variants of lipid acyl transferases with preferred characteristics. Suitable methods for
15 performing 'shuffling' can be found in EP0 752 008, EP1 138 763, EP1 103 606. Shuffling can also be combined with other forms of DNA mutagenesis as described in US 6,180,406 and WO 01/34835.

Thus, it is possible to produce numerous site directed or random mutations into a
20 nucleotide sequence, either *in vivo* or *in vitro*, and to subsequently screen for improved functionality of the encoded polypeptide by various means. Using *in silico* and *exo* mediated recombination methods (see WO 00/58517, US 6,344,328, US 6,361,974), for example, molecular evolution can be performed where the variant produced retains very low homology to known enzymes or proteins. Such variants thereby obtained
25 may have significant structural analogy to known transferase enzymes, but have very low amino acid sequence homology.

As a non-limiting example, In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wild type or other
30 mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide.

The application of the above-mentioned and similar molecular evolution methods allows the identification and selection of variants of the enzymes of the present invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate

As will be apparent to a person skilled in the art, using molecular evolution tools an enzyme may be altered to improve the functionality of the enzyme.

Suitably, the lipid acyltransferase used in the invention may be a variant, i.e. may contain at least one amino acid substitution, deletion or addition, when compared to a parental enzyme. Variant enzymes retain at least 1%, 2%, 3%, 5%, 10%, 15%, 20%, 30%, 40%, 50 %, 60%, 70%, 80%, 90%, 95%, 97%, 99% homology with the parent enzyme. Suitable parent enzymes may include any enzyme with esterase or lipase activity. Preferably, the parent enzyme aligns to the pfam00657 consensus sequence.

In a preferable embodiment a variant lipid acyltransferase enzyme retains or incorporates at least one or more of the pfam00657 consensus sequence amino acid residues found in the GDSx, GANDY and HPT blocks.

Enzymes, such as lipases with no or low lipid acyltransferase activity in an aqueous environment may be mutated using molecular evolution tools to introduce or enhance the transferase activity, thereby producing a lipid acyltransferase enzyme with significant transferase activity suitable for use in the compositions and methods of the present invention.

Suitably, the lipid acyltransferase for use in the invention may be a variant with enhanced enzyme activity on polar lipids, preferably phospholipids and/or glycolipids when compared to the parent enzyme. Preferably, such variants also have low or no activity on lyso polar lipids. The enhanced activity on polar lipids, phospholipids and/or glycolipids may be the result of hydrolysis and/or transferase activity or a combination of both.

Variant lipid acyltransferases for use in the invention may have decreased activity on triglycerides, and/or monoglycerides and/or diglycerides compared with the parent enzyme.

Suitably the variant enzyme may have no activity on triglycerides and/or monoglycerides and/or diglycerides.

Alternatively, the variant enzyme for use in the invention may have increased activity on triglycerides, and/or may also have increased activity on one or more of the following, polar lipids, phospholipids, lecithin, phosphatidylcholine, glycolipids, digalactosyl monoglyceride, monogalactosyl monoglyceride.

Variants of lipid acyltransferases are known, one or more of such variants may be suitable for use in the methods and uses of the invention. For example, variants of lipid acyl transferases are described in the following references:

Hilton S, Buckley JT. Studies on the reaction mechanism of a microbial lipase/acyltransferase using chemical modification and site-directed mutagenesis. J Biol Chem. 1991 Jan 15;266(2):997-1000.

Robertson DL, Hilton S, Wong KR, Koepke A, Buckley JT. Influence of active site and tyrosine modification on the secretion and activity of the *Aeromonas hydrophila* lipase/acyltransferase. J Biol Chem. 1994 Jan 21;269(3):2146-50.

Brumlik MJ, Buckley JT. Identification of the catalytic triad of the lipase/acyltransferase from *Aeromonas hydrophila*. *J Bacteriol.* 1996 Apr;178(7):2060-4.

- 5 Peelman F, Vinaimont N, Verhee A, Vanloo B, Verschelde JL, Labeur C, Seguret-Mace S, Duverger N, Hutchinson G, Vandekerckhove J, Tavernier J, Rosseneu M. A proposed architecture for lecithin cholesterol acyl transferase (LCAT): identification of the catalytic triad and molecular modeling. *Protein Sci.* 1998 Mar;7(3):587-99.

10 AMINO ACID SEQUENCES

The present invention also encompasses amino acid sequences of polypeptides having the specific properties as defined herein.

- 15 As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide".

The amino acid sequence may be prepared/isolated from a suitable source, or it may be
20 made synthetically or it may be prepared by use of recombinant DNA techniques.

Suitably, the amino acid sequences may be obtained from the isolated polypeptides taught herein by standard techniques.

- 25 One suitable method for determining amino acid sequences from isolated polypeptides is as follows:

Purified polypeptide may be freeze-dried and 100 µg of the freeze-dried material may be dissolved in 50 µl of a mixture of 8 M urea and 0.4 M ammonium hydrogen
30 carbonate, pH 8.4. The dissolved protein may be denatured and reduced for 15 minutes at 50°C following overlay with nitrogen and addition of 5 µl of 45 mM dithiothreitol. After cooling to room temperature, 5 µl of 100 mM iodoacetamide may be added for

the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

135 μ l of water and 5 μ g of endoproteinase Lys-C in 5 μ l of water may be added to the
5 above reaction mixture and the digestion may be carried out at 37°C under nitrogen for 24 hours.

The resulting peptides may be separated by reverse phase HPLC on a VYDAC C18 column (0.46x15cm;10 μ m; The Separation Group, California, USA) using solvent A:
10 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides may be re-chromatographed on a Develosil C18 column using the same solvent system, prior to N-terminal sequencing. Sequencing may be done using an Applied Biosystems 476A sequencer using pulsed liquid fast cycles according to the manufacturer's instructions (Applied Biosystems, California, USA).

15

SEQUENCE IDENTITY OR SEQUENCE HOMOLOGY

The present invention also encompasses the use of sequences having a degree of
20 sequence identity or sequence homology with amino acid sequence(s) of a polypeptide having the specific properties defined herein or of any nucleotide sequence encoding such a polypeptide (hereinafter referred to as a "homologous sequence(s)"). Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can
25 be equated with "identity".

The homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of the enzyme.

30

In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98%

identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express
5 homology in terms of sequence identity.

In the present context, a homologous sequence is taken to include a nucleotide sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to a nucleotide sequence encoding a polypeptide of the present invention (the
10 subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express
15 homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

20 % homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

25 Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is
30 performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and

deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

5 However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most
10 commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for
15 a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package
20 (Devereux *et al* 1984 Nuc. Acids Research 12 p387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al* 1999 Short Protocols in Molecular Biology, 4th Ed - Chapter 18), FASTA (Altschul *et al* 1990 J. Mol. Biol. 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online
25 searching (see Ausubel *et al* 1999, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

30

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a

scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public
5 default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

- 10 Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244).

Once the software has produced an optimal alignment, it is possible to calculate %
15 homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent
20 substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino
25 acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below.
30 Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β -alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α -carbon substituent group is on the residue's nitrogen atom rather than the α -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

Nucleotide sequences for use in the present invention or encoding a polypeptide having the specific properties defined herein may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences.

The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences discussed herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid
5 sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and
10 will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence
15 changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction polypeptide recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer,
20 e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40
25 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in
30 the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

- 5 Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain
- 10 reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

15 HYBRIDISATION

The present invention also encompasses sequences that are complementary to the sequences of the present invention or sequences that are capable of hybridising either to the sequences of the present invention or to sequences that are complementary

20 thereto.

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR)

25 technologies.

The present invention also encompasses the use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the subject sequences discussed herein, or any derivative, fragment or derivative thereof.

The present invention also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences discussed herein.

Hybridisation conditions are based on the melting temperature (T_m) of the nucleotide
5 binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning
Techniques, Methods in Enzymology, Vol. 152, Academic Press, San Diego CA), and
confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about $T_m - 5^\circ\text{C}$ (5°C below the T_m of the
10 probe); high stringency at about 5°C to 10°C below T_m ; intermediate stringency at
about 10°C to 20°C below T_m ; and low stringency at about 20°C to 25°C below T_m .
As will be understood by those of skill in the art, a maximum stringency hybridisation
can be used to identify or detect identical nucleotide sequences while an intermediate
(or low) stringency hybridisation can be used to identify or detect similar or related
15 polynucleotide sequences.

Preferably, the present invention encompasses sequences that are complementary to
sequences that are capable of hybridising under high stringency conditions or
intermediate stringency conditions to nucleotide sequences encoding polypeptides
20 having the specific properties as defined herein.

More preferably, the present invention encompasses sequences that are complementary
to sequences that are capable of hybridising under high stringent conditions (e.g. 65°C
and $0.1\times\text{SSC}$ { $1\times\text{SSC} = 0.15\text{ M NaCl}$, $0.015\text{ M Na-citrate pH } 7.0$ }) to nucleotide
25 sequences encoding polypeptides having the specific properties as defined herein.

The present invention also relates to nucleotide sequences that can hybridise to the
nucleotide sequences discussed herein (including complementary sequences of those
discussed herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

- 5 Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridising to the nucleotide sequences discussed herein under conditions of intermediate to maximal stringency.

- 10 In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

- 15 In a more preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

EXPRESSION OF POLYPEPTIDES

- 20 A nucleotide sequence for use in the present invention or for encoding a polypeptide having the specific properties as defined herein can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence, in polypeptide form, in and/or from a compatible host cell. Expression may be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in
25 eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

- 30 The polypeptide produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal

sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

EXPRESSION VECTOR

5

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

Preferably, the expression vector is incorporated in the genome of the organism. The term "incorporated" preferably covers stable incorporation into the genome.

10

The nucleotide sequence of the present invention or coding for a polypeptide having the specific properties as defined herein may be present in a vector, in which the nucleotide sequence is operably linked to regulatory sequences such that the regulatory sequences are capable of providing the expression of the nucleotide sequence by a suitable host organism, i.e. the vector is an expression vector.

15

The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide having the specific properties as defined herein.

20

The choice of vector, e.g. plasmid, cosmid, virus or phage vector, will often depend on the host cell into which it is to be introduced.

The vectors may contain one or more selectable marker genes – such as a gene which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Alternatively, the selection may be accomplished by co-transformation (as described in WO91/17243).

25

Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

30

Thus, in a further embodiment, the invention provides a method of making nucleotide sequences of the present invention or nucleotide sequences encoding polypeptides having the specific properties as defined herein by introducing a nucleotide sequence into a replicable vector, introducing the vector into a compatible host cell, and growing
5 the host cell under conditions which bring about replication of the vector.

The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.
10

REGULATORY SEQUENCES

In some applications, a nucleotide sequence for use in the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined
15 herein may be operably linked to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

20 The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with
25 the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

30 The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

Enhanced expression of the nucleotide sequence encoding the enzyme having the specific properties as defined herein may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions.

5

Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.

Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial, fungal or yeast host are well known in the art.

10

CONSTRUCTS

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence encoding a polypeptide having the specific properties as defined herein for use according to the present invention directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

15

20

The construct may even contain or express a marker which allows for the selection of the genetic construct.

25

For some applications, preferably the construct comprises at least a nucleotide sequence of the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined herein operably linked to a promoter.

30

HOST CELLS

The term "host cell" - in relation to the present invention includes any cell that comprises either a nucleotide sequence encoding a polypeptide having the specific properties as defined herein or an expression vector as described above and which is used in the recombinant production of a polypeptide having the specific properties as defined herein.

Thus, a further embodiment of the present invention provides host cells transformed or transfected with a nucleotide sequence of the present invention or a nucleotide sequence that expresses a polypeptide having the specific properties as defined herein. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells. Preferably, the host cells are not human cells.

Examples of suitable bacterial host organisms are gram negative bacterium or gram positive bacteria.

Depending on the nature of the nucleotide sequence encoding a polypeptide having the specific properties as defined herein, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a different fungal host organism should be selected.

The use of suitable host cells, such as yeast, fungal and plant host cells - may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

The host cell may be a protease deficient or protease minus strain.

ORGANISM

- 5 The term "organism" in relation to the present invention includes any organism that could comprise a nucleotide sequence according to the present invention or a nucleotide sequence encoding for a polypeptide having the specific properties as defined herein and/or products obtained therefrom.
- 10 Suitable organisms may include a prokaryote, fungus, yeast or a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises a nucleotide sequence coding for a polypeptide having the specific properties as defined herein and/or the products obtained therefrom, and/or
15 wherein a promoter can allow expression of the nucleotide sequence coding for a polypeptide having the specific properties as defined herein within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

The term "transgenic organism" does not cover native nucleotide coding sequences in
20 their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, a nucleotide sequence coding for a
25 polypeptide having the specific properties as defined herein, constructs as defined herein, vectors as defined herein, plasmids as defined herein, cells as defined herein, or the products thereof. For example the transgenic organism can also comprise a nucleotide sequence coding for a polypeptide having the specific properties as defined herein under the control of a heterologous promoter.

TRANSFORMATION OF HOST CELLS/ORGANISM

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

5

Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as

10

by removal of introns.

In another embodiment the transgenic organism can be a yeast.

15

Filamentous fungi cells may be transformed using various methods known in the art - such as a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

20

Another host organism can be a plant. A review of the general techniques used for transforming plants may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

25

General teachings on the transformation of fungi, yeasts and plants are presented in following sections.

TRANSFORMED FUNGUS

A host organism may be a fungus - such as a filamentous fungus. Examples of suitable such hosts include any member belonging to the genera *Thermomyces*, *Acremonium*, *Aspergillus*, *Penicillium*, *Mucor*, *Neurospora*, *Trichoderma* and the like.

- 5 Teachings on transforming filamentous fungi are reviewed in US-A-5741665 which states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N. crassa* is found, for example in Davis and de Serres, *Methods Enzymol* (1971) 17A: 79-143.

10

Further teachings on transforming filamentous fungi are reviewed in US-A-5674707.

In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

15

A transgenic *Aspergillus* according to the present invention can also be prepared by following, for example, the teachings of Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R. (Editors) *Aspergillus: 50 years on. Progress in industrial microbiology* vol 29. Elsevier Amsterdam 1994. pp. 641-666).

20

Gene expression in filamentous fungi has been reviewed in Punt *et al.* (2002) *Trends Biotechnol* 2002 May;20(5):200-6, Archer & Peberdy *Crit Rev Biotechnol* (1997) 17(4):273-306.

25 TRANSFORMED YEAST

In another embodiment, the transgenic organism can be a yeast.

30 A review of the principles of heterologous gene expression in yeast are provided in, for example, *Methods Mol Biol* (1995), 49:341-54, and *Curr Opin Biotechnol* (1997) Oct;8(5):554-60

In this regard, yeast – such as the species *Saccharomyces cerevisiae* or *Pichia pastoris* (see FEMS Microbiol Rev (2000 24(1):45-66), may be used as a vehicle for heterologous gene expression.

- 5 A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", *Yeasts*, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).
- 10 For the transformation of yeast, several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen *et al.*, (1978, *Proceedings of the National Academy of Sciences of the USA* 75, 1929); Beggs, J D (1978, *Nature*, London, 275, 104); and Ito, H *et al* (1983, *J Bacteriology* 153, 163-168).
- 15 A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as but not limited to yeast species such as *Pichia* sp., *Hansenula* sp or *Kluyveromyces*, *Yarrowinia* species or a species of *Saccharomyces* including *Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyce* such as, for
- 20 example, *S. pombe* species.

A strain of the methylotrophic yeast species *Pichia pastoris* can be used as the host organism.

- 25 In one embodiment the host organism is a *Hansenula* species, such as *Hansenula polymorpha* (as described in WO01/38544).

The transformed yeast cells may be selected using various selective markers – such as auxotrophic markers dominant antibiotic resistance markers.

TRANSFORMED PLANTS/PLANT CELLS

A host organism suitable for the present invention may be a plant. A review of the general techniques may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27), or in WO01/16308. The transgenic plant may produce enhanced levels of phytosterol esters and phytostanol esters, for example.

Therefore the present invention also relates to a method for the production of a transgenic plant with enhanced levels of phytosterol esters and phytostanol esters, comprising the steps of transforming a plant cell with a lipid acyltransferase as defined herein (in particular with an expression vector or construct comprising a lipid acyltransferase as defined herein), and growing a plant from the transformed plant cell.

15 SECRETION

Often, it is desirable for the polypeptide to be secreted from the expression host into the culture medium from where the enzyme may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*glaA* - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the a-factor gene (yeasts e.g. *Saccharomyces*, *Kluyveromyces* and *Hansenula*) or the α -amylase gene (*Bacillus*).

DETECTION

A variety of protocols for detecting and measuring the expression of the amino acid sequence are known in the art. Examples include enzyme-linked immunosorbent

assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays.

A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures.

10

Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3,817,837; US-A-3,850,752; US-A-3,939,350; US-A-3,996,345; US-A-4,277,437; US-A-4,275,149 and US-A-4,366,241.

15

Also, recombinant immunoglobulins may be produced as shown in US-A-4,816,567.

20 FUSION PROTEINS

A polypeptide having the specific properties as defined herein may be produced as a fusion protein, for example to aid in extraction and purification thereof. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the protein sequence.

25

Gene fusion expression systems in *E. coli* have been reviewed in Curr. Opin. Biotechnol. (1995) 6(5):501-6.

30

In another embodiment of the invention, the amino acid sequence of a polypeptide having the specific properties as defined herein may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a
5 chimeric substance expressing a heterologous epitope that is recognised by a commercially available antibody.

The invention will now be described, by way of example only, with reference to the following Figures and Examples.

10

Figure 1 shows a pfam00657 consensus sequence from database version 6 (SEQ ID No. 1);

Figure 2 shows an amino acid sequence (SEQ ID No. 2) obtained from the organism
15 *Aeromonas hydrophila* (P10480; GI:121051);

Figure 3 shows an amino acid sequence (SEQ ID No. 3) obtained from the organism *Aeromonas salmonicida* (AAG098404; GI:9964017);

20 Figure 4 shows an amino acid sequence (SEQ ID No. 4) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number NP_631558);

Figure 5 shows an amino acid sequence (SEQ ID No. 5) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number: CAC42140);
25

Figure 6 shows an amino acid sequence (SEQ ID No. 6) obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number P41734);

Figure 7 shows an alignment of selected sequences to pfam00657 consensus sequence;
30

Figure 8 shows a pairwise alignment of SEQ ID No. 3 with SEQ ID No. 2 showing 93% amino acid sequence identity. The signal sequence is underlined. + denotes

differences. The GDSX motif containing the active site serine 16, and the active sites aspartic acid 116 and histidine 291 are highlighted (see shaded regions). Numbers after the amino acid is minus the signal sequence;

5 Figure 9 shows a nucleotide sequence (SEQ ID No. 7) encoding a lipid acyl transferase according to the present invention obtained from the organism *Aeromonas hydrophila*;

Figure 10 shows a nucleotide sequence (SEQ ID No. 8) encoding a lipid acyl transferase according to the present invention obtained from the organism *Aeromonas*
10 *salmonicida*;

Figure 11 shows a nucleotide sequence (SEQ ID No. 9) encoding a lipid acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number
15 NC_003888.1:8327480..8328367);

Figure 12 shows a nucleotide sequence (SEQ ID No. 10) encoding a lipid acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number
20 AL939131.1:265480..266367);

Figure 13 shows a nucleotide sequence (SEQ ID No. 11) encoding a lipid acyl transferase according to the present invention obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number Z75034);
25

Figure 14 shows an amino acid sequence (SEQ ID No. 12) obtained from the organism *Ralstonia* (Genbank accession number: AL646052);

Figure 15 shows a nucleotide sequence (SEQ ID No. 13) encoding a lipid acyl transferase according to the present invention obtained from the organism *Ralstonia*;
30

Figure 16 shows SEQ ID No. 20. Scoe1 NCBI protein accession code CAB39707.1
GI:4539178 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 17 shows a nucleotide sequence shown as SEQ ID No. 21 encoding NCBI
5 protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein
[Streptomyces coelicolor A3(2)];

Figure 18 shows an amino acid shown as SEQ ID No.22. Scoe2 NCBI protein
accession code CAC01477.1 GI:9716139 conserved hypothetical protein
10 [Streptomyces coelicolor A3(2)];

Figure 19 shows a nucleotide sequence shown as SEQ ID No. 23 encoding Scoe2
NCBI protein accession code CAC01477.1 GI:9716139 conserved hypothetical
protein [Streptomyces coelicolor A3(2)];

15 Figure 20 shows an amino acid sequence (SEQ ID No.24) Scoe3 NCBI protein
accession code CAB88833.1 GI:7635996 putative secreted protein. [Streptomyces
coelicolor A3(2)];

20 Figure 21 shows a nucleotide sequence shown as SEQ ID No. 25 encoding Scoe3
NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein.
[Streptomyces coelicolor A3(2)];

Figure 22 shows an amino acid sequence (SEQ ID No.26) Scoe4 NCBI protein
25 accession code CAB89450.1 GI:7672261 putative secreted protein. [Streptomyces
coelicolor A3(2)];

Figure 23 shows a nucleotide sequence shown as SEQ ID No. 27 encoding Scoe4
NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein.
30 [Streptomyces coelicolor A3(2)];

Figure 24 shows an amino acid sequence (SEQ ID No.28) Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [*Streptomyces coelicolor* A3(2)];

- 5 Figure 25 shows a nucleotide sequence shown as SEQ ID No. 29, encoding Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [*Streptomyces coelicolor* A3(2)];

- Figure 26 shows an amino acid sequence (SEQ ID No.30) Srim1 NCBI protein
10 accession code AAK84028.1 GI:15082088 GDSL-lipase [*Streptomyces rimosus*];

Figure 27 shows a nucleotide sequence shown as SEQ ID No. 31 encoding Srim1 NCBI protein accession code AAK84028.1 GI:15082088 GDSL-lipase [*Streptomyces rimosus*];

15

Figure 28 shows an amino acid sequence (SEQ ID No.32)A lipid acyl transferase from *Aeromonas hydrophila* (ATCC #7965);

- Figure 29 shows a nucleotide sequence (SEQ ID No. 33) encoding a lipid
20 acyltransferase from *Aeromonas hydrophila* (ATCC #7965);

Figure 30 shows an amino acid sequence (SEQ ID No.34) of a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

- 25 Figure 31 shows a nucleotide sequence (SEQ ID No 35) encoding a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

- Figure 32 shows that homologues of the *Aeromonas* genes can be identified using the basic local alignment search tool service at the National Center for Biotechnology Information, NIH, MD, USA and the completed genome databases. The GDSX motif
30 was used in the database search and a number of sequences/genes potentially encoding enzymes with lipolytic activity were identified. Genes were identified from the genus *Streptomyces*, *Xanthomonas* and *Ralstonia*. As an example below, the *Ralstonia*

solanacearum was aligned to the *Aeromonas salmonicida* (satA) gene. Pairwise alignment showed 23% identity. The active site serine is present at the amino terminus and the catalytic residues histidine and aspartic acid can be identified;

5 Figure 33 shows the Pfam00657.11 [family 00657, database version 11] consensus sequence (hereafter called Pfam consensus) and the alignment of various sequences to the Pfam consensus sequence. The arrows indicate the active site residues, the underlined boxes indicate three of the homology boxes indicated by [Upton C and Buckley JT (1995) Trends Biochem Sci 20; 179-179]. Capital letters in the Pfam
10 consensus indicate conserved residues in many family members. The - symbol indicates a position where the hidden Markov model of the Pfam consensus expected to find a residue but did not, so a gap is inserted. The . symbol indicates a residue without a corresponding residue in the Pfam consensus. The sequences are the amino acid sequences listed in Figures 16, 18, 20, 22, 24, 26, 28 and 30.

15 Figure 34 shows the Pfam00657.11 [family 00657, database version 11] consensus sequence (hereafter called Pfam consensus) and the alignment of various sequences to the Pfam consensus sequence. The arrows indicate the active site residues, the underlined boxes indicate three of the homology boxes indicated by [Upton C and
20 Buckley JT (1995) Trends Biochem Sci 20; 179-179]. Capital letters in the Pfam consensus indicate conserved residues in many family members. The - symbol indicates a position where the hidden Markov model of the Pfam consensus expected to find a residue but did not, so a gap is inserted. The . symbol indicates a residue without a corresponding residue in the Pfam consensus. The sequences are the amino
25 acid sequences listed in Figures 2, 16, 18, 20, 26, 28 and 30. All these proteins were found to be active against lipid substrates.

Figure 35 shows a expression vector pet12-AsalGCAT= pSM containing the C-terminal His-tagged *Aeromonas salmonicida* lipid acyltransferase gene;

30

Figure 36 shows the results of testing cell extracts in a NEFA Kit Assay, which depicts the activity of a recombinant, *A. salmonicida* lipid acyltransferase, towards lecithin.

The wells from left to right indicate: a positive control, a negative control (i.e. extracts from empty plasmid) and samples collected after 0, 1, 2 and 3 hours cultivation after IPTG induction;

- 5 Figure 37 shows growth optimisation of BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM showing cultivation at 30 °C resulted in the production of enzyme with high activity towards lecithin. Cell extracts were tested for phospholipase activity using the NEFA kit assay. Wells from left to right: positive control; negative control; 20°C; 30°C;

10

- Figure 38 shows crude cell extracts from BL21(DE3)pLysS expressing active lipid acyltransferase incubated with the substrate lecithin and reaction mixture was analyzed using thin layer chromatography showing the presence of degradation products. Lanes: 1. No enzyme; 2. + A.sal -10ul 37°C; 3. + A. sal -20ul 37°C; 4. + A.sal - 15 10ul 24°C; 5. + A. sal -20u 24°C;

Figure 39 shows partial purification of the *Aeromonas salmonicida* Acyl Transferase showing the phospholipase activity associated with purified His-tag protein. SE = Sonicated extracts, His = Purified with Ni-NTA spin-kit from Qiagen;

20

Figure 40 shows the expression vector pet12-A.h. GCAT=pSMa containing the C-terminal His-tagged *Aeromonas hydrophila* Glycerolipid Acyl Transferase (GCAT) gene was used to transform *E.coli* strain BL21(DE3)pLysS;

- 25 Figure 41 shows the activity of the crude extracts (5 & 10ul) containing the recombinant *Aeromonas hydrophila* GCAT enzyme was tested towards lecithin using Non-Esterified Fatty Acid (NEFA) kit (Roche, Switzerland), showing the presence of active enzyme towards the phospholipid, lecithin;

- 30 Figure 42 shows growth optimisation of BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM showing cultivation at 30 °C resulted in the production

of enzyme with high activity towards lecithin. Cell extracts were tested for phospholipase activity using the NEFA kit assay;

Figure 43 shows the partial purification of the *Aeromonas hydrophila* & *A. salmonicida* Acyl Transferases showing the phospholipase activity associated with purified His-tag protein. SE = Sonicated extracts,
His = Purified with Ni-NTA spin-kit from Qiagen);

Figure 44 shows the expression of the *Aeromonas* genes in *Bacillus subtilis* 163 showing the production of secreted enzyme with activity towards both lecithin and DGDG. pUB-AH= construct containing the *A. hydrophila* gene and pUB-AS, construct with the *A. salmonicida* gene, Culture filtrate was incubated with the substrates for 60 minutes.

Figure 45 and Figure 46 show graphs depicting fatty acid and cholesterol ester as a function of time. The graphs depict results obtained for GLC analysis in the assay for measurement of acyltransferase activity in a foodstuff using lecithin and cholesterol in buffer as substrate;

Figure 47 shows an amino acid sequence (SEQ ID No. 36) of the fusion construct used for mutagenesis of the *Aeromonas hydrophila* lipid acyltransferase gene in Example 17. The underlined amino acids is a xylanase signal peptide;

Figure 48 shows a nucleotide sequence (SEQ ID No. 54) encoding an enzyme from *Aeromonas hydrophila* including a xylanase signal peptide;

Figure 49 shows the structure of protein-fatty acid condensates of amino acids;

Figure 50 shows a schematic representing the reaction between a fatty acid from phosphatidylcholine when transferred to the free hydroxyl group of amino acids having a free hydroxyl group available for esterification, e.g. tyrosine or serine; and

Figure 51 shows a schematic of the reaction between DGDG and glucose, which is catalysed by a lipid acyltransferase.

EXAMPLES

5

EXAMPLE 1: The cloning, sequencing and heterologous expression of a transferase from *Aeromonas salmonicida* subsp. *Salmonicida*

Strains used:

- 10 *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC 14174) was obtained from ATCC and grown overnight at 30°C in Luria-Bertani medium (LB). The cells were centrifuged and genomic DNA was isolated using the procedures for genomic DNA isolation from Qiagen Ltd. Genomic DNA buffer set (cat.19060), protease K (cat. 19131) and RNase A (cat. 19101) were all obtained from Qiagen Ltd. (Boundary
15 court Gatwick Court, West Sussex, RH10 2AX).

- Host bacterial strain BL21(DE3)pLysS (Novagen) was used for production of the recombinant *Aeromonas* enzymes. Competent cells of BL21(DE3)pLysS were used as host for transformation with the expression vector **pet12-AsalGCAT=pSM**.
20 Transformants containing the appropriate plasmid were grown at 37 °C in LB agar medium containing 100-ug ampicillin/ml.

Construction of expression vector **pet12-AsalGCAT- pSM**:

25

- For all DNA amplifications of the transferase genes from *Aeromonas*, genomic DNA (0.2-1 ul) was used as template and *pfu* DNA polymerase (2.5 units) was used with 10ul of 10x *pfu* buffer, 1ul each primer (50pmol/ul), 200 uM dNTP in a total reaction volume of 100ul. PCR reactions were performed in a programmable thermal cycler
30 using the following conditions: 95 °C for 30 seconds, 30 cycles of 95 °C for 30 seconds, 60 °C for 1 minute and 68 °C for 2 minutes. An additional extension of 5 minutes at 72 °C was applied.

The PCR amplification of the transferase gene from *A. salmonicida* was carried in 2 separate PCR reactions. PCR reaction 1 was performed using primer pairs, as1USNEW(5'AGCATATGAAAA AATGGTTTGT TTGTTTATTG GGG 3' [SEQ ID No. 36]) and asls950new (5' GTG ATG GTG GGC GAG GAA CTC GTA CTG3' [SEQ ID No. 37]). A second PCR reaction was performed to incorporate a C-terminal Histidine tag using the PCR product from the first reaction and the primers: as1USNEW(5'AGCATATGAAAA AATGGTTTGT TTGTTTATTG GGG 3' [SEQ ID No. 38]) and AHLS1001(5'TTGGATCC GAATTCAT CAATG GTG ATG GTG ATG GTG GGC3' [SEQ ID No. 39]). The PCR product from the second reaction was purified and digested with restriction enzymes NdeI and BamHI. 2 ug of pET 12a vector DNA was also digested with restriction enzymes NdeI and BamHI and treated with phosphatase. The restriction enzyme-treated pet12a and PCR product from reaction 2 were purified and ligated using the Rapid Ligation Kit (Roche, Switzerland). The ligation mix was used to transform *E. coli* TOP10 cells. Transformants were plated on LB agar medium containing 100ug/ml ampicillin.

The T7 promoter primer (5'TAATACGACTCACTATAG3' [SEQ ID No. 40]) and the T7 terminator primer (5'CTAGTTATTGCTCAGCGG3' [SEQ ID No. 41]) were used to verify the sequences and the orientation of the cloned transferase genes in pET12a vector. DNA sequencing was performed using ABI Prism® BigDye™ Terminators Cycle sequencing kit with 500ng plasmid DNA as template and 3.2pmol T7 promoter and terminator primers.

The construct shown in Figure 35 was used to transform competent bacterial host strain BL21(DE3)pLysS (Novagen) and ampicillin resistant transformants were picked and used for expression analysis.

Expression of the recombinant *Aeromonas salmonicida* lipid acyltransferase

Quantification of enzyme activity towards lecithin was determined on cell extracts using Non-Esterified Fatty Acid (NEFA) kit (Roche, Switzerland).

In Figure 36, BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT=pSM was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at 37°C until OD₆₀₀ = 0.6 to 1.0 is reached. The cultures are then induced using IPTG (0.4mM) and incubation was continued for the next 3 hours. Samples were taken at 0 hour, 1, 2, and 3 hours after IPTG induction. Enzyme Activity was tested using the NEFA kit and lecithin as substrate.

Growth Optimisation for the production of more active enzymes

BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT=pSM was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at different growth temperatures (37°C, 30 °C, & 20 °C). The optimal condition for the production of active lipid acyltransferase enzyme was when cultures are grown at 30°C as shown in Figure 37.

Partial purification of recombinant *Aeromonas salmonicida* transferase

Strain BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT=pSM was grown at 37°C & crude cell extracts were prepared by sonication. The recombinant enzyme was further purified from the sonicated crude cell extracts using the Ni-NTA spin kit from Qiagen. Phospholipase activity using the NEFA kit & Lecithin as substrate. Crude cell extracts from BL21(DE3)pLysS expressing active transferase incubated with the substrate lecithin and reaction mixture was analysed using thin layer chromatography showing the presence of degradation products (see Figure 38).

Partial Purification of recombinant *Aeromonas salmonicidae* transferase. Strain BL21(DE3)pLysS harbouring the expression vector pet12-AsalGCAT=pSM was grown at 37°C and crude cell extracts were prepared by sonication. The recombinant enzyme were further purified from the sonicated crude cell extract using the Ni-NTA spin kit from Qiagen. Phospholipase activity using the NEFA kit and lecithin as substrate was tested (see Figure 39).

EXAMPLE 2 Cloning and Expression of *Aeromonas hydrophila* transferase in *E. coli*

5

Aeromonas hydrophila (ATCC # 7965) was obtained from ATCC and grown overnight at 30°C in Luria-Bertani medium (LB). The cells were centrifuged and genomic DNA was isolated using the procedures for genomic DNA isolation from Qiagen Ltd. Genomic DNA buffer set (cat.19060), protease K (cat. 19131) and RNase A (cat. 19101) were all obtained from Qiagen Ltd. (Boundary court Gatwick Court, West Sussex, RH10 2AX).

Host bacterial strain BL21(DE3)pLysS (Novagen) was used for production of the recombinant *Aeromonas* enzymes. Competent cells of BL21(DE3)pLysS were used as host for transformation with the expression vector pet12a-A.h.GCAT=pSMa. Transformants containing the appropriate plasmid were grown at 37 °C in LB agar medium containing 100-ug ampicillin/ml.

Construction of expression vector pet12a-A.h.GCAT- pSMa:

For all DNA amplifications of the transferase gene from *Aeromonas*, genomic DNA (0.2-1 ul) was used as template and *pfu* DNA polymerase (2.5 units) was used with 10ul of 10x *pfu* buffer, 1ul each primer (50pmol/ul), 200 uM dNTP in a total reaction volume of 100ul. PCR reactions were performed in a programmable thermal cycler using the following conditions: 95 °C for 30 seconds, 30 cycles of 95 °C for 30 seconds, 60 °C for 1 minute and 68 °C for 2 minutes. An additional extension of 5 minutes at 72 °C was applied.

The PCR amplification of the transferase gene from *A. hydrophila* (ATCC # 7965) was carried out in 2 separate PCR reactions.

30

PCR reaction 1 was performed using primer pairs, AHUS1 (5'GTCATATGAAAAAATGGTTTGTGTGTTTATTGGGATTGGTC3', SEQ ID No.

42) and ahls950 (5'ATGGTGATGGTGGGCGAGGAACTCGTACTG3', SEQ ID No. 43).

A second PCR reaction was performed to incorporate a C-terminal Histidine tag using the PCR product from the first reaction and the primer pairs:

AHUS1(5'GTCATATGAAAAAATGGTTTGTGTGTTTATTGGGATTGGTTC3' SEQ ID No. 44,) and AHLS1001(5'TTGGATCCGAATTCATCAATGGTGATGGTGATGGTGGGC3' SEQ ID No. 45).

The PCR product from the second reaction was purified and digested with restriction enzymes NdeI and BamHI. 2 ug of pET 12a vector DNA was also digested with restriction enzymes NdeI and BamHI and treated with phosphatase. The restriction enzyme-treated pet12a and PCR product from reaction 2 were purified and ligated using the Rapid Ligation Kit (Roche, Switzerland). The ligation mix was used to transform *E. coli* TOP10 cells. Transformants were plated on LB agar medium containing 100ug/ml ampicillin.

The T7 promoter primer (5'TAATACGACTCACTATAG3') and the T7 terminator primer (5'CTAGTTATTGCTCAGCGG3') were used to verify the sequences and the orientation of the cloned GCAT genes in pET12a vector. DNA sequencing was performed using ABI Prism® BigDye™ Terminators Cycle sequencing kit with 500ng plasmid DNA as template and 3.2pmol T7 promoter and terminator primers.

The construct shown in Figure 40 was used to transform competent bacterial host strain BL21 (DE3)pLysS (Novagen) and ampicillin resistant transformants were picked and used for expression analysis.

30 Expression of the *Aeromonas hydrophila* transferase in BL21(DE3)pLysS

The *E. coli* strain BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT= pSma was grown in LB medium + 100ug/ml ampicillin and incubated

with shaking at 37°C until OD₆₀₀ = 0.6 to 1.0 is reached. The cultures are then induced using IPTG (0.4mM) and incubation was continued for the next 3 hours. Samples were taken at 0hour, 1, 2, and 3 hours after IPTG induction. Enzyme Activity was tested using the NEFA kit and lecithin as substrate (Figure 41).

5

Growth Optimisation for the production of more active enzymes

BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT=pSMA was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at different growth temperatures (37°C, 30 °C, & 20 °C). The optimal condition for the production of active GCAT enzyme was when cultures are grown at 30°C as shown in Figure 42.

10

Partial purification of recombinant *A.hydrophila* transferase (GCAT)

Strain BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT=pSMA was grown at 37°C & crude cell extracts were prepared by sonication. The recombinant enzyme was further purified from the sonicated crude cell extracts using the Ni-NTA spin kit from Qiagen. Phospholipase activity assay using the NEFA kit & Lecithin as substrate. (Figure 43).

15

EXAMPLE 3: Expression of *Aeromonas* transferases in *Bacillus subtilis* 163

Plasmid Construction

Two different *Bacillus subtilis* expression vectors (pUB 110 & pBE5) were used for the heterologous expression of the *Aeromonas* genes in *Bacillus subtilis*. The pUB110 vector contains the alpha amylase promoter while the pBE vector has the P32 promoter as the regulatory region for the expression of the fused *Aeromonas* genes. In pUB110, the first amino acid of the mature GCAT genes of *Aeromonas* were fused in frame with the last amino acid of the xylanase signal peptide sequence from *Bacillus subtilis* via the restriction site NheI, creating an additional 2 amino acids in front of the mature proteins. pBE5 contains the cgtase signal sequence fusion at the NcoI site for secretion of the recombinant proteins into the culture filtrate.

25

PCR reactions were carried out to obtain the *Aeromonas* genes fuse in frame to the signal sequences of the pUB 110 and the pBE5 vectors. PCRs were performed using the following primer pairs for *A. hydrophila* gene:

- 5 PCR reaction 1: usAH_{ncol} (5'ATGCCATGGCCGACAGCCGTCCCGCC3', SEQ ID No. 46) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 47)

PCR reaction 2: US-Ah_{nheI} (5'TTGCTAGCGCCGACAGCCGTCCCGCC3', SEQ ID No. 48.) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 49)

10

PCRs were performed using the following primer pairs for *A. salmonicida* gene:

PCR reaction 3: US-As_{ncol} (5'TTGCCATGGCCGACACTCGCCCCGCC3', SEQ ID No. 50) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 51)

15

PCR reaction 4: US-AS_{nheI} (5'TTGCTAGCGCCGACACTCGCCCCGCC3', SEQ ID No. 52) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 53)

- 20 All the PCR products were cloned into PCR blunt II (TOPO vector) and sequenced with reverse & forward sequencing primers.

- Clones from PCR reactions 1 & 3 were cut with NcoI & Bam HI and used as inserts for ligation to the pBE5 vector cut with NcoI/BamHI/phosphatase. Clones from PCR
25 reactions 2 & 4 were cut with NheI & Bam HI and used as inserts for ligation to the pUB vector that was cut with NheI/BamHI/phosphatase.

- 30 **Expression of the *Aeromonas* transferase genes in *Bacillus subtilis* and characterization of the enzyme activity.**

The acyl transferases from the two *Aeromonas* species have been successfully expressed in *E. coli* (results above). The *Bacillus* pUB110 & pBE5 gene fusion

constructs were used to transform *Bacillus subtilis* and transformants were selected by plating on kanamycin plates. The kanamycin resistant transformants isolated and grown in 2xYT are capable of heterologous expression of the *Aeromonas* genes in *Bacillus*. The culture filtrates have digalactosyldiacylglycerol (DGDG) galactolipase activity, in addition to having both acyl transferase and phospholipase activities. The activity towards digalactosyldiacylglycerol (DGDG) was measured after 60 minutes of incubation of culture supernatant with the substrate, DGDG from wheat flour (obtainable from Sigma) as well as the activity towards lecithin as shown in Figure 44. *Bacillus* produced the enzyme after overnight (20-24 hours) to 48 hours of cultivation in the culture medium as a secreted protein. In some instances, the expression of the *Aeromonas* genes has been shown to interfere with cell viability and growth in *Bacillus* & *E. coli*, it is therefore necessary to carefully select expression strains and optimise the growth conditions to ensure expression. For example, several *Bacillus* host strains (B.s 163, DB104 and OS 21) were transformed with the expression vectors for growth comparison. B.s163 is transformable with the 2 *Aeromonas* genes and is capable of expressing active protein. DB104 is transformable with all the constructs but is only able to express *A. salmonicida* transferase.

EXAMPLE 4: Fermentation and Purification of *Aeromonas* lipid acyltransferases produced in *E.coli*

***E.coli* Fermentations:**

Microorganisms

Two strains of *Escherichia coli*, one containing an *Aeromonas hydrophila* (Example 2) lipid acyltransferase and two containing *Aeromonas salmonicida* lipid acyltransferases, (Example 1) were used in this study.

The *E. coli* strain containing the *A. hydrophila* gene was named DIDK0124, and the *E. coli* strain containing the *A. salmonicida* gene was named DIDK0125. The fermentation with DIDK0124 was named HYDRO0303 and the fermentation with

DIDK0125 was named SAL0302. The purified protein from HYDRO025 was named REF#138. The purified protein from HYDRO0303 was named REF#135.

Growth media and culture conditions

5 LB-agar

The LB agar plates used for maintaining the strains contained: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar, 100 mg/L ampicillin and 35 mg/L chloramphenicol. The agar plates were incubated at 30°C.

10 LB shake flask

The LB medium (50 mL pr shake flask) used for production of inoculum material for the bioreactor cultivations contained: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 100 mg/L ampicillin and 35 mg/L chloramphenicol. The shake flasks were inoculated from the LB agar plates, and incubated at 30°C and 200 rpm.

15

Bioreactor cultivation

The bioreactor cultivations were carried out in 6 L in-house built bioreactors filled with 4 L medium containing: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 8 g/L KH_2PO_4 , 0.9 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 40 g/L glucose monohydrate, 0.4 mL/ ADD APT®
20 Foamstop Sin 260 (ADD APT Chemicals AG, Helmond, The Netherlands), 10 mg/L $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.7 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 3 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3 mg/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 10 mg/L EDTA, 0.1 mg/L $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 0.1 mg/L CoCl_2 , 0.1 mg/L H_3BO_4 , 0.1 mg/L KI, 0.1 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1 g/L ampicillin and 35 mg/L chloramphenicol.

25

The bioreactors were inoculated with an amount of LB culture ensuring end of growth after approximately 20 hours of cultivation (calculated from the maximum specific growth rate of 0.6 h^{-1} , the OD_{600} of the LB shake flask and the final OD_{600} in the bioreactor of approximately 20).

30

SAL0302 was inoculated with 10 mL of LB culture, and HYDRO0303 was inoculated with 4 mL of LB culture.

The bioreactors were operated at the following conditions: temperature 30°C, stirring
5 800-1000 rpm (depending on experiment), aeration 5 L/min, pH 6.9, pH control 8.75% (w/v) NH₃-water and 2 M H₂SO₄. Induction was achieved by addition of isopropyl β-D-thiogalactoside to a final concentration of 0.6 mM, when 0.4 moles (HYDRO0303) and 0.7 moles CO₂ was produced respectively.

10 Harvest

The following procedure was used for harvest and homogenisation of the biomass:

- 1) The fermentation broth from the fermentations was centrifuged at 5000 × g and 4°C for 10 minutes, and the supernatant was discharged. The biomass was stored at -20°C until use. The biomass was thawed and resuspended in 500 mL
15 of 20 mM NaH₂PO₄, pH 7.4, 500 mM NaCl, 10 mM Imidazole and Complete (EDTA-free) protease inhibitor (Roche, Germany).
- 2) The suspended biomass was homogenized at 2 kbar and 4°C in a cell disrupter from Constant Systems Ltd (Warwick, UK).
- 3) The cell debris was removed by centrifugation at 10.000 × g and 4°C for 30
20 minutes followed by collection of the supernatant.
- 4) The supernatant was clarified further by centrifugation at 13.700× g and 4°C for 60 minutes, followed by collection of the supernatant.
- 5) The supernatant was filtered through 0.2 μm Vacu Cap filters (Pall Life Sciences, UK) and the filtrate was collected for immediate chromatographic
25 purification.

Chromatographic purification of the Transferases

A column (2.5 x 10 cm) was packed with 50 ml of Chelating Sepharose ff. gel and charged with Ni-sulphate (according to the method described by manufacturer,
30 Amersham Biosciences). The column was equilibrated with 200 ml of 20 mM NaH₂PO₄, pH 7.4, 500 mM NaCl, 10 mM Imidazole. 400 ml of crude was applied to the column at a flow rate of 5 ml/min. The column was then washed with 20 mM

NaH₂PO₄, pH 7.4, 500 mM NaCl, 10 mM Imidazole until the UV₂₈₀ reached the base line. The GCAT was then eluted with 40 ml of 20 mM NaH₂PO₄, pH 7.4, 500 mM NaCl and 500 mM Imidazole.

5 **EXAMPLE 5: Fermentation and Purification of *Aeromonas* lipid acyltransferases produced in *Bacillus subtilis*.**

Fermentations

BAC0318-19, BAC0323-24

10 **Microorganism**

The microorganisms used in this study originate from transformation of a *Bacillus subtilis* host strain, #163 with a plasmid containing the gene encoding the *Aeromonas salmonicida* transferase inserted in the vector pUB110OIS. The expression of the gene is controlled by an alpha-amylase promoter, and the secretion of the transferase is
15 mediated by the *B. subtilis* xylanase signal sequence (Example 3). The strains were named DIDK0138 (fermentation BAC0318-19) and DIDK0153 (fermentation BAC0323-24).

Growth media and culture conditions

20 **Pre culture medium**

A shake flask (500 mL total volume, with baffles) was added 100 mL of a medium containing:

NaCl	5 g/L
K ₂ HPO ₄	10 g/L
25 Soy flour	20 g/L
Yeast extract, BioSpringer 106	20 g/L
Antifoam, SIN260	5 mL/L

pH was adjusted to 7.0 before autoclaving

30

After autoclaving 6 mL 50% (w/w) Nutriose were added pr flask. Kanamycin was added at a concentration of 50 mg/L after autoclaving.

Inoculation

A pre culture shake flask was inoculated with frozen culture directly from a 25% (w/v) glycerol stock. The shake flask was incubated at 33°C and 175 rpm for approximately 5 16 hours, whereupon 50 mL was used to inoculate the fermentor.

Fermentations

The fermentations were carried out in 6 L in house built fermentors.

The batch medium (3 L) contained:

10	Corn steep liquor (50% dw)	40 g/L
	Yeast extract BioSpringer 153 (50% dw)	10 g/L
	NaCl	5 g/L
	CaCl ₂ , 2H ₂ O	0.25 g/L
	Mn(NO ₃) ₂ , H ₂ O	0.2 g/L
15	Antifoam SIN260	1 mL/L
	Kanamycin (filter sterilised to the fermentor after autoclaving	50 mg/L

The feed contained:

20	Glucose monohydrate	540 g/kg
	MgSO ₄ , 7H ₂ O	4.8 g/kg
	Antofoam SIN260	4 mL/kg
25	Yeast extract, BioSpringer 153 (50% dw) (autoclaved separately)	150 g/kg

The feed in fermentation BAC0318 and BAC0323 was started based on the accumulated CO₂, according to the equations below:

Feed – flow[g/h] = 0, $\text{AcCO}_2 < 0.15$

Feed – flow[g/h] = $2.85 + t \cdot 1.54$, $\text{AcCO}_2 \geq 0.15$ and $t < 12$

Feed – flow[g/h] = 21.3, $t > 12$

t: time (hours) from the point when the accumulated CO_2 (AcCO_2) reached 0.15 moles.

The feed in fermentation BAC0319 and BAC0324 was started based on the
5 accumulated CO_2 , according to the equations below:

Feed – flow[g/h] = 0, $\text{AcCO}_2 < 0.15$

Feed – flow[g/h] = $2.0 + t \cdot 1.08$, $\text{AcCO}_2 \geq 0.15$ and $t < 12$

Feed – flow[g/h] = 15, $t > 12$

t: time (hours) from the point when the accumulated CO_2 (AcCO_2) reached 0.15 moles.

10 The pH was controlled at 7.0 by adding 12.5% (w/v) NH_3 -water or 2M phosphoric acid.

The aeration was 3 L/min corresponding to 1 vvm.

The temperature was 33°C.

The fermentor was equipped with two 8 cm Ø Rushton impellers placed with a distance of 10
15 cm.

Harvest

The biomass was removed by centrifugation at 16,000× g for 10 minutes at room temperature. The supernatant was filter sterilized, and the filtrate was used for
20 purification and application tests.

EXAMPLE 6 : The “Transferase Assay in Buffered Substrate” for measurement of acyltransferase activity of an enzyme.

25 The lipid acyltransferase was isolated from *Aeromonas salmonicida* and expressed in *Bacillus subtilis*. This enzyme is very efficient in transferring fatty acid from lecithin

to cholesterol during formation of cholesterol esters. It has also been shown that the enzyme has some hydrolytic activity, which is observed by the formation of free fatty acid. Traditional phospholipases (EC3.1.1.4 and EC3.1.1.32) have the ability to hydrolyse lecithin during formation of free fatty acids and lysolecithin, and no transferase reactions has been reported for these enzymes.

We detail herein an assay that is able to measure both transferase and hydrolytic activity of enzymes and thus to identify lipid acyltransferases in accordance with the present invention, the assay uses a substrate which contains lecithin and cholesterol. In this work a substrate based on phosphatidylcholine and cholesterol dispersed in a buffer was used. Quantification of reaction products was made by extraction of lipids from the substrate followed by GLC analysis of the lipid components.

Procedure

15

Materials

L-alpha-Phosphatidylcholine 95% (Plant) Avanti no. 441601

Cholesterol: Sigma cat. C 8503

Cholesteryl Palmitate, Sigma C 6072

20 Cholesteryl Stearate, Sigma C 3549

HEPES buffer Sigma cat. No. H 3375

Chloroform, Analytical grade.

Enzymes

25 Purified GCAT from *A. salmonicida* #178-9

TLC analysis.

TLC-plate was activated in a heat cupboard (110°C) for ½ h.

100 ml running buffer was poured into a chromatography chamber with lid. The walls of the chamber were covered with filter paper (Whatman 2) in order to saturate the chamber with the solvent vapour.

The TLC-plate was placed in a frame and the sample was applied onto the TLC plate 2 cm from the bottom. The TLC plate was then placed in the TLC chamber with the running buffer. When the running buffer reached 14 cm from the bottom of the plate, the TLC plate was taken out and dried in fume board, and then placed in the heat cupboard at 110 °C for 10 minutes.

The TLC-plate was then immersed in the developing reagent, and dried in the heat cupboard at 110 °C for 15 minutes

Running-buffer:

- 10 Nr. IV: Chloroform : Methanol : H₂O (65:25:4)
Nr. I: P-ether : MTBE : Acetic acid (60:40:1)

Developing buffer (Vanadate-buffer):

- 15 32 g Na₂CO₃ ad 300 ml H₂O (1M)
18.2 g vanadate pentoxide (V₂O₅) is added and dissolved during gentle heating.
The solution is cooled to ambient.
Carefully 460 ml 2.5 M H₂SO₄. (460 ml H₂O +61 ml H₂SO₄) is added
Water is added to 1000 ml.

20

GLC analysis

- Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25 mm ID x 0.1 µ film thickness 5% phenyl-methyl-silicone (CP Sil 8 CB from Chrompack).

25 Carrier gas: Helium.

Injector. PSSI cold split injection (initial temp 50°C heated to 385°C), volume 1.0µl

Detector FID: 395°C

- | | | | |
|---------------------------|----|-----|-----|
| Oven program: | 1 | 2 | 3 |
| 30 Oven temperature, °C. | 90 | 280 | 350 |
| Isothermal, time, min. | 1 | 0 | 10 |
| Temperature rate, °C/min. | 15 | 4 | |

Sample preparation: 30 mg of sample was dissolved in 9 ml Heptane:Pyridin, 2:1 containing internal standard heptadecane, 0.5 mg/ml. 300µl sample solution was transferred to a crimp vial, 300 µl MSTFA (N-Methyl-N-trimethylsilyl-trifluoroacetamid) was added and reacted for 20 minutes at 60°C.

Calculation: Response factors for mono-di-triglycerides and free fatty acid are determined from Standard 2 (mono-di-triglyceride). The response factors for Cholesterol, Cholesteryl Palmitate and Cholesteryl Stearate were determined from pure reference materials.

Results: Transferase assay based on phosphatidylcholine and cholesterol as substrate.

In the following the transferase activity of the transferase was tested in a substrate based on phosphatidylcholine and cholesterol according to the following procedure.

450 mg phosphatidylcholine (>95% PC Avanti item no. 441601) and 50 mg cholesterol was dissolved in chloroform and evaporated to dryness under vacuum. 300 mg cholesterol/phosphatidylcholine mixture was transferred to a Wheaton glass and 15 ml 50mM HEPES buffer pH 7 was added. The lipid was dispersed in the buffer during agitation.

The substrate was heated to 35 °C during mixing with a magnetic stirrer and 0.25 ml enzyme solution was added. This is a very high water environment of approximately 95% water.

25

Samples of 2 ml were taken out after 0, 5, 10, 15, 25, 40 and 60 minutes reaction time. Immediately 25 µl 4M HCl was added to acidify the free fatty acid and stop the enzyme reaction. 3.00 ml chloroform was added, and the sample was shaken vigorously on a Whirley for 30 seconds. The sample was centrifuged and 2 ml of the chloroform phase was isolated and filtered through 0.45-µm filters into a 10 ml tared Dram glass. The chloroform was evaporated under a stream of nitrogen at 60°C, and the samples were scaled again. The extracted lipid was analysed by GLC.

The results from the GLC analysis are shown in Table 1. The results are expressed in % calculated on extracted lipid. The amount of fatty acid and cholesterol ester formed as a function of time is illustrated in Figure 45. It can be concluded from Figure 45 that the enzyme reaction is not linear as a function of time, because an initially strong both hydrolytic and transferase activity is observed. After approximately 10 minutes and until approximately 60 minutes the reaction shows an almost linear response of fatty acid and cholesterol ester formation as a function of time. It was therefore decided to look at the enzymatic reaction in this time interval.

Table 1

Minutes	0	5	10	15	25	40	60
Cholesterol, %	10.064	8.943	8.577	8.656	8.102	7.856	7.809
Cholesterol ester, %	0.000	1.571	2.030	2.058	2.282	2.659	3.081
FFA total, %	0.260	1.197	1.239	1.466	2.445	2.943	3.940

From the knowledge about the amount of lipid in the reaction mixture and the amount of enzyme added it was possible to calculate the formation of fatty acid and cholesterol ester expressed in $\mu\text{mol/ml}$ enzyme (Table 2 and Figure 46).

Table 2

Minutes	10	15	25	40	60
	$\mu\text{mol/ml}$	$\mu\text{mol/ml}$	$\mu\text{mol/ml}$	$\mu\text{mol/ml}$	$\mu\text{mol/ml}$
FFA total	58.1	68.7	114.6	138.0	184.7
Cholesterol ester	88.8	90.0	99.3	115.6	133.8

From the results in Table 2 and the slope of the curves in Figure 46 it was possible to calculate the amount of fatty acid and cholesterol ester as a function of time expressed in $\mu\text{mol}/\text{min}$ per ml enzyme.

5

The calculation of the hydrolytic activity and the transferase activity is shown in Table 3. The relative transferase activity was determined using the protocol for the determination of % acyltransferase activity as described hereinbefore.

10 Table 3

Hydrolytic activity (fatty acid)	2.52	$\mu\text{mol}/\text{min}$ per ml enzyme
Transferase activity(cholesterol ester)	0.94	$\mu\text{mol}/\text{min}$ per ml enzyme
Total activity	3.45	$\mu\text{mol}/\text{min}$ per ml enzyme
Relative Transferase activity	27.1	%
Relative hydrolytic activity	72.9	%

Screening of other enzymes for transferase activity.

- 15 The method mentioned above was used to screen different lipolytic enzymes for transferase and hydrolytic activity. The enzymes were tested as shown in Table 4.

Table 4

		1	2	3	4	5
Substrate	ml	15	15	15	15	15
#178-9Transferase A. salmonicida 32 PLU-7/ml	ml	0.25				
5% #3016, LIOPAN [®] F (F. oxysporum)	ml		0.25			
5%, Thermomyces lanuginosus	ml			0.25		

5% <i>Candida rugosa</i> #2983	ml				0.25	
5% <i>Candida cylindracea</i> #3076	ml					0.25

The substrate containing 300mg phosphatidylcholine/cholesterol dispersed in 50 mM HEPES buffer pH 7.0 was heated to 35 °C with agitation. Enzyme solution was added and the sample was kept at 35 °C with agitation. Samples were taken out with regular interval and extracted with Chloroform. The isolated lipids were analysed by GLC with results shown in Table 5.

Table 5

Sample								
1	Transferase 178-9							
	Minutes	0	5	10	15	25	40	60
	FFA	1.216	2.516	2.983	2.62	2.894	3.448	3.911
	Cholesterol	7.547	6.438	6.365	6.15	6.136	5.936	5.662
	Chl. Ester	0	1.835	2.177	2.44	2.58	2.851	3.331
2	<i>Fusarium oxysporum</i> (LIOPAN® F)	0	5	10	15	25	40	60
	FFA	1.216	1.345	1.796	1.95	2.487	2.424	2.977
	Cholesterol	7.547	7.309	7.366	7.33	7.429	7.341	7.326
	Chl. Ester	0	0.26	0.386	0.35	0.267	0.36	0.394
3	<i>Thermomyces lanuginosus</i>	0	5	10	15	25	40	60
	FFA	1.216	0.853	0.875	1	0.896	1.105	1.009
	Cholesterol	7.547	7.384	7.639	7.63	7.675	7.603	7.529
	Chl. Ester	0	0	0	0	0	0	0
4	<i>Candida rugosa</i> (#2938)	0	5	10	15	25	40	60
	FFA	1.216	0.982	0.987	1.02	1.135	1.131	1.15

	Cholesterol	7.547	7.438	7.656	7.66	7.638	7.575	7.585
	Chl. Ester	0	0	0	0	0	0	0
5	Candida cylandracea (#3076)	0	5	10	15	25	40	60
	FFA	1.216	1.032	1.097	1.07	1.203	1.131	1.43
	Cholesterol	7.547	7.502	7.425	7.65	7.619	7.502	7.411
	Chl. Ester	0	0	0	0	0	0	0

From the GLC analysis it was observed that only the lipid acyltransferase (178-9) produced significant amount of cholesterol ester and fatty acids. Phospholipase from *Fusarium oxysporum* also gave a steady increase in free fatty acid but only an initial small amount formation of cholesterol ester was formed but no increase in cholesterol ester as a function of time was observed.

Based on the knowledge about the amount of lipid substrate and the GLC analyses it was possible to calculate the relative transferase activity and the relative hydrolytic activity based on the results from 10 to 60 minutes reaction time. The results from Transferase 178-9 and *Fusarium oxysporum* lipase are shown in Table 6. The other enzymes tested showed no activity.

15 Table 6

	Transferase 178-9	<i>Fusarium oxysporum</i>
Hydrolytic activity, micromole/min per ml enzyme	1.03	0.96
Transferase activity, micromole/min per ml enzyme	0.40	0.01
Total activity, micromole/min per ml enzyme	1.43	0.98
Relative hydrolytic activity	71.8	98.7

Relative transferase activity	28.2	1.3
-------------------------------	------	-----

5 The result shown in Table 6 confirm a significant transferase activity from the lipid acyltransferase (sample 178-9). It is also observed that the relative transferase activity is in good agreement with the experiment mentioned in Table 3.

10 A very low transferase activity form *Fusarium oxysporum* phospholipase is however observed. This transferase level is so low that it falls within the uncertainty of the analysis. As expected *Fusarium oxysporum* phospholipase has a significant hydrolytic activity.

Conclusion.

15 An artificial substrate based on purified phosphatidylcholine and cholesterol was used as a substrate to measure the activity of transferase from *Aeromonas salmonicida*. Between 10 minutes and 60 minutes reaction time the assay gave an almost linear formation of free fatty acids and cholesterol ester as a function of time. Based on the activity between 10 and 60 minutes reaction time the hydrolytic activity and the transferase activity was calculated.

20 Based on the results from the assay of the lipid acyltransferase (in this instance a GCAT) from *Aeromonas salmonicida* in a artificial substrate of phosphatidylcholine/cholesterol in buffer it is concluded that this enzyme has very good transferase activity also in a system with a very high water content.

25 The phosphatidylcholine/cholesterol in buffer assay, can be used to measure the transferase and hydrolytic activity of an enzyme. The phosphatidylcholine/cholesterol in buffer is only linear within a certain time limit.

30 **EXAMPLE 7: Immobilisation of a lipid acyltransferase from *Aeromonas salmonicida***

A lipid acyltransferase (in this instance a GCAT) from *A. salmonicida* was immobilised on Celite 535 535 (from Fluka) by acetone precipitation. 10 ml enzyme solution in 20 mM TEA buffer pH 7 was agitated slowly with 0,1 gram Celite 535 535 (from Fluka) for 2 hours at room temperature.

50ml cool acetone was added during continued agitation.

The precipitate was isolated by centrifugation 5000 g for 1 minute.

The precipitate was washed 2 times with 20 ml cold acetone.

The Celite was tried at ambient temperature for about 1 hour

10

The enzyme has also been shown to have a high activity in environments with high water content (6- 89 %)water environments, the use of the transferase, and other transferases for use in the invention can therefore also be used in immobilised enzyme applications with a significant water content. This allows the replacement of the solvents used by the current immobilised lipases in the bioconversion of lipids using transferases.

EXAMPLE 8: Variants of a lipid acyltransferase from *Aeromonas hydrophila* (Ahyd2) (SEQ ID No. 36 (see Figure 47))

20

Mutations were introduced using the QuikChange® Multi-Site Directed Mutagenesis kit from Stratagene, La Jolla, CA 92037, USA following the instructions provided by Stratagene.

25 Variants at Tyr256 showed an increased activity towards phospholipids.

Variants at Tyr256 and Tyr260 showed an increased activity towards galactolipids.

Variants at Tyr265 show an increased transferase activity with galactolipids as the acyl donor.

30

The numbers indicate positions on the following sequence: An enzyme from *Aeromonas hydrophila* the amino acid sequence of which is shown as SEQ ID No. 36 in Figure 47 (the underlined amino acids show a xylanase signal peptide). The nucleotide sequence is as shown as SEQ ID No. 54 in Figure 48.

5

EXAMPLE 9 "Assay in Low Water Environment"

Transferase reactions of lipolytic enzymes in low water environment .

10 Procedure

Materials.

Cholesterol Sigma cat. C 8503

L-alpha-Phosphatidylcholine 95% (Plant) Avanti #441601

15 Soybean oil, Aarhus United, DK.

Chloroform, Analytical grade

Enzymes.

#179, GCAT from *A. salmonicida*

20 #2427, Phospholipase A1 from *Fusarium oxysporum*. LIPOPAN® F from Novozymes, Denmark

#1991, Phospholipase A2 from Pancreas, LIPOMOD 22L from Biocatalysts, UK

#2373, *Candida Antarctica* lipase, Novozyme 525 L from Novozymes Denmark.

25

Enzyme assay

13.1 % Lecithin and 6.6% cholesterol was dissolved in soybean oil by heating to 60 °C during agitation

The substrate was scaled in a 20ml Wheaton glass and heated to 46 °C

Water and enzyme solution was added and a stopwatch is started.

30 At regular intervals 50 mg samples were transferred to a 10ml Dram glass and frozen.
The isolated lipids were analysed by GLC

GLC analysis

For GLC analysis protocols – see example 6

5 Results

The experiment was set up as shown in Table 8.

The substrate based on soybean oil containing 13.1 % lecithin and 6.6% cholesterol was heated to 46°C. The enzyme solution was added and a stopwatch started.

10 After 30, 60 and 120 minutes reaction time samples were taken out for GLC analysis.

Table 8

		1	2	3	4	5
Substrate	Gram	5	5	5	5	5
Transferase #179-C72, 56 PLU-7/ml	MI		0.3			
#2427, 200 PLU-7/ml	MI			0.3		
Pancreas PLA 2 #1991 6300 PLU/ml	MI				0.3	
Novozyme 525 L, #2373, 200 LIPU/ml	MI					0.3
Water	MI	0.3				
% water		6	6	6	6	6

15 The results from the GLC analysis is shown in Table 9. The results are expressed in percent based total sample composition. Based on the GLC results it was possible to calculate the amount of fatty acid and cholesterol ester produced by enzymatic reaction relative to the control sample without enzyme added. Under these experimental conditions the total enzymatic activity was estimated as the hydrolytic

20 activity measured as free fatty acid formation and the transferase activity estimated as cholesterol ester formation. From these results and the information about molecular

weight of fatty acid and cholesterol ester it was possible to calculate to relative molar hydrolytic activity and the relative molar transferase activity as shown in Table 10.

Table 9

Enzyme	Reaction time minutes	Cholesterol		
		Fatty acid %	1 %	Cholesterol ester %
Control	120	0.533	7.094	0.000
#179	30	0.770	5.761	2.229
#179	60	0.852	5.369	2.883
#179	120	0.876	4.900	3.667
#2427	30	3.269	7.094	0.000
#2427	60	3.420	7.094	0.000
#2427	120	3.710	7.094	0.000
#1991	30	2.871	7.094	0.000
#1991	60	3.578	7.094	0.000
#1991	120	3.928	7.094	0.000
#2373	30	1.418	7.094	0.000
#2373	60	1.421	7.094	0.000
#2373	120	1.915	7.094	0.000

5

Table 10

Enzyme	Reaction time minutes	Fatty acid produced	Cholesterol Used	Cholesterol ester produced	Hydrolytic	Transferase
					activity %	Activity %
#179	30	0.238	1.334	2.229	20	80
#179	60	0.319	1.725	2.883	21	79

#179	120	0.343	2.195	3.667	18	82
#2427	30	2.737	0.000	0.000	100	0
#2427	60	2.887	0.000	0.000	100	0
#2427	120	3.177	0.000	0.000	100	0
#1991	30	2.338	0.000	0.000	100	0
#1991	60	3.046	0.000	0.000	100	0
#1991	120	3.395	0.000	0.000	100	0
#2373	30	0.885	0.000	0.000	100	0
#2373	60	0.888	0.000	0.000	100	0
#2373	120	1.383	0.000	0.000	100	0

Conclusion

In these experiments it was observed that all the tested enzymes showed hydrolytic activity because the amount of fatty acid increased. However the only enzyme which showed transferase activity was GCAT from *A. salmonicida*. It is therefore concluded that in an oily system with lecithin and cholesterol containing 6% water phospholipase A1 from *Fusarium oxysporum*, phospholipase A2 from *pancreas* and a lipase from *Candida antarctica* only showed hydrolytic activity.

Example 10: Carbohydrate ester production with immobilised lipid acyltransferase according to the present invention.

Carbohydrate esters of fatty acids like sucrose esters and glucose esters are traditionally produced by the reaction of a fatty acid or a fatty acid soap and the carbohydrate at high temperature (Journal of the American Oil Chemists' Society (1978) 55; 4; 398-401) This procedure however has the disadvantage of forming side reactions and coloured by-products.

In the present invention carbohydrate esters of fatty acids are produced by a transferase reaction using lecithin as fatty acid donor and a carbohydrate like glucose as acceptor molecule.

The reaction is conducted in a flow reactor with a lipid acyl transferase immobilised on a solid support.

Procedure.

- 5 100 gram glucose is dissolved in 1000 ml water during agitation then 200 gram phosphatidylcholine is dispersed in the water phase during agitation and heated to 40°C.

pH is adjusted to pH 6.5.

- A flow reactor is packed with 100 g of a lipid acyltransferase from *A. salmonicida*
10 immobilised on a solid support.

The flow reactor is placed in a heating cabinet at 40 °C.

The reaction mixture is pumped into the column with 2 ml / min.

The reaction product is collected.

- The water in the reaction product is removed by thin film vacuum evaporation and the
15 lipids isolated.

The glucose ester is separated from the other lipids by solvent fractionation.

Carbohydrate esters can be used for many applications, such as efficient emulsifiers within the food and non-food industry

20

Example 11 – Protein ester production with a lipid acyltransferase according to the present invention.

- In the present invention fatty-acid condensates of amino acids, peptides or proteins are
25 produced by a transferase reaction. In this reaction phosphatidylcholine is used as donor for the transfer of fatty acid to the free hydroxyl group of amino acids (such as tyrosine, serine or threonine) having a free hydroxyl group available for esterification.

Procedure 1.

- 30 50 gram l-tyrosine (or serine or threonine) is dissolved in 1000 ml water during agitation then 200 gram phosphatidylcholine is dispersed in the water phase during agitation and heating to 40°C.

pH is adjusted to pH 7 and kept at this pH with NaOH or HCl.

50 ml of the lipid acyltransferase enzyme from *A. salmonicida* is added and the reaction is continued at 40 °C with agitation.

Samples are taken out at regular intervals and analysed by TLC and HPLC.

- 5 After 20 h reaction time the reaction has reached equilibrium and the reaction is stopped.

Tyrosine fatty acid condensate, lecithin and lysolecithin are isolated from the reaction media by centrifugation according to standard methods (see "Centrifuges, Filtering" in Ullmann's Encyclopedia of Industrial Chemistry for example (2002) by Wiley-VCH Verlag GmbH & Co. KGaA).

15 Tyrosine fatty acid condensate is further purified by hydrophobic interaction column chromatography and the fraction containing tyrosine fatty acid condensate is isolated and the solvent removed by evaporation. (see 'Basic Principles of Chromatography' in Ullmann's Encyclopedia of Industrial Chemistry (2002) by Wiley-VCH Verlag GmbH & Co. KGaA.)

Procedure 2.

- 20 In the following the transferase activity of a lipid acyltransferase is tested in a substrate based on phosphatidylcholine and l-tyrosine according to the following procedure.

450 mg phosphatidylcholine (>95% PC Avanti item no. 441601) and 50 mg l-tyrosine is scaled in a Wheaton glass and 15 ml 50mM HEPES buffer pH 7 is added. The lipid is dispersed in the buffer during agitation.

The substrate is heated to 35 °C whilst mixing with a magnetic stirrer and 0.25 ml Transferase 10 PLU/ml is added.

- 30 Samples of 2 ml are taken out after 0, 5, 10, 15, 25, 40 and 60 minutes reaction time.

Immediately 25 μ l 4M HCl is added to acidify the free fatty acid and stop the enzyme reaction. 3.00 ml chloroform is added, and the sample is shaken vigorously on a Whirley for 30 seconds. The sample is centrifuged and 2 ml of the chloroform phase is isolated and filtered through 0.45- μ m filters into a 10 ml tared Dram glass.

5

The chloroform is evaporated under a stream of nitrogen at 60°C, and the sample is scaled again. The extracted lipid is analysed by TLC .

10 **Example 12 – Hydroxy acid ester (in particular lactic acid ester) production with a lipid acyltransferase according to the present invention.**

Hydroxy esters of fatty acids are traditionally produced by the reaction between a fatty acid and a hydroxy acid at high temperature using an inorganic salts or metal ions as catalysts (see for example Bailey's Industrial Oil and Fat Products, Fifth edition, 15 Volume 3. Edible Oil and Fat Products: Products and Application Technology, page 502-511.) This procedure however has the disadvantage of forming side reactions and coloured by-products.

In the present invention hydroxy acid esters of fatty acids are produced by a 20 transferase reaction using lecithin as fatty acid donor and a hydroxy acid (in particular lactic acid) as acceptor molecule.

Procedure.

50 gram lactic is dissolved in 1000 ml water whilst agitating, then 200 gram 25 phosphatidylcholine is dispersed in the water phase during agitation and heated to 40°C.

pH is adjusted to pH 6.5 and kept at this pH with NaOH or HCl.

30 50 ml of lipid acyltransferase enzyme from *A. salmonicida* is added and the reaction is continued at 40 °C whilst agitating.

Samples are taken out at regular intervals and analysed by TLC and GLC.

After 20 h reaction time the reaction has reached equilibrium and the reaction is stopped.

5

Lactic acid ester, lecithin and lysolecithin are isolated from the reaction media by centrifugation according to standard methods (see "Centrifuges, Filtering" in Ullmann's Encyclopedia of Industrial Chemistry for example (2002) by Wiley-VCH Verlag GmbH & Co. KGaA).

10

Lactic acid ester is further purified by molecular distillation and a lactic acid ester of fatty acid with high purity is obtained.

15 **Example 13 –Citric acid ester production with a lipid acyltransferase according to the present invention.**

Transferase assay based on phosphatidylcholin and citric acid as substrate.

20 In the following the transferase activity of lipid acyl transferase from *A. salmonicida* is tested in a substrate based on phosphatidylcholin and citric acid according to the following procedure.

25 450 mg phosphatidylcholine (>95% PC Avanti item no. 441601) and 50 mg citric acid is scaled in a Wheaton glass and 15 ml 50mM HEPES buffer pH 7 is added. The lipid is dispersed in the buffer during agitation.

The substrate is heated to 35 °C during mixing with a magnetic stirrer and 0.25 ml lipid acyltransferase from *A. salmonicida* 10 PLU/ml is added.

30 Samples of 2 ml are taken out after 0, 5, 10, 15, 25, 40 and 60 minutes reaction time.

Immediately 25 μ l 4M HCl is added to acidify the free fatty acid and stop the enzyme reaction. 3.00 ml chloroform is added, and the sample is shaken vigorously on a Whirley for 30 seconds. The sample is centrifuged and 2 ml of the chloroform phase is isolated and filtered through 0.45- μ m filters into a 10 ml tared Dram glass.

5

The chloroform is evaporated under a stream of nitrogen at 60°C, and the sample is scaled again. The extracted lipid is analysed by TLC .

10

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific

15

embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

Danisco A/S
Langebrogade 1
DK-1001 Copenhagen
Denmark

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the
DEPOSITOR:

Escherichia coli
TOP10pPet12aAhydro

Accession number given by the
INTERNATIONAL DEPOSITARY AUTHORITY:

NCIMB 41204

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐

a scientific description

☒

a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on
22 December 2003 (date of the original deposit)¹

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on
(date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received
by it on

(date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd.,

Address: 23 St Machar Drive
Aberdeen
AB24 3RY
Scotland, UK.

Signature(s) of person(s) having the power to represent the
International Depositary Authority or of authorised
official(s):

Terence Dando
Date: 9 January 2004

¹ Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was
acquired.
Form BP/4 (sole page)

**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

Danisco A/S
Langebrogade 1
DK-1001 Copenhagen
Denmark

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: AS ABOVE Address:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 41204 Date of the deposit or of the transfer ¹ : 22 December 2003
III. VIABILITY STATEMENT	
<p>The viability of the microorganism identified under II above was tested on 22 December 2003 ². On that date, the said microorganism was:</p> <p><input checked="checked" type="checkbox"/> ³ viable</p> <p><input type="checkbox"/> no longer viable</p>	

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

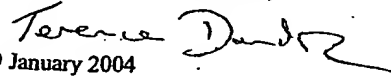
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd.,

Address: 23 St Machar Drive
Aberdeen
AB24 3RY
ScotlandSignature(s) of person(s) having the power
to represent the International Depositary
Authority or of authorised official(s):

Date: 9 January 2004

⁴ Fill in if the information has been requested and if the results of the test were negative.

105

**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

Danisco A/S
Langebrogade 1
DK-1001 Copenhagen
Denmark

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

NAME AND ADDRESS OF DEPOSITOR**I. IDENTIFICATION OF THE MICROORGANISM**

Identification reference given by the
DEPOSITOR:

Escherichia coli
TOP10pPet12aAsalmo

Accession number given by the
INTERNATIONAL DEPOSITARY AUTHORITY:

NCIMB 41205

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐

a scientific description

☒

a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

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International Depositary Authority or of authorised
official(s):

Terence Dando

Date: 9 January 2004

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identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 41205
Address:	Date of the deposit or of the transfer ¹ : 22 December 2003
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 22 December 2003 ² . On that date, the said microorganism was:	
<input checked="checked" type="checkbox"/> ³	viable
<input type="checkbox"/> ³	no longer viable

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴

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Name: NCIMB Ltd.,

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Aberdeen
AB24 3RY
ScotlandSignature(s) of person(s) having the power
to represent the International Depositary
Authority or of authorised official(s):

Date: 9 January 2004



⁴ Fill in if the information has been requested and if the results of the test were negative.

CLAIMS

1. A method of producing one or more of a carbohydrate ester, a protein ester, a protein subunit ester, a hydroxy acid ester, which method comprises admixing
5 an acyl donor, an acyl acceptor and water to produce a high water environment comprising 5-98% water, wherein said acyl donor is a lipid substrate selected from one or more of the group consisting of a phospholipid, a lysophospholipid, a triacylglyceride, a diglyceride, a glycolipid or a lysoglycolipid and said acyl acceptor is selected from one or more of the group
10 consisting of a carbohydrate, a protein, a protein subunit, or a hydroxy acid; and contacting the admixture with a lipid acyltransferase, such that said lipid acyltransferase catalyses one or both of the following reactions: alcoholysis or transesterification.
2. A method according to claim 1 wherein the lipid acyltransferase is
15 immobilised.
3. A method according to claim 1 or claim 2 wherein the method comprises purifying the carbohydrate ester, protein ester, protein subunit ester, hydroxy acid ester.
4. A method according to any one of claims 1-3 wherein the lipid acyltransferase
20 is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.
5. A method according to any one of the preceding claims wherein the lipid
25 acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.
6. A method according to any one of the preceding claims wherein the lipid
30 acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*,

Campylobacter, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas* and *Candida*.

7. A method according to any one of the preceding claims wherein the lipid acyltransferase comprises one or more of the following amino acid sequences:
- 5 (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix) the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, or an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32 or SEQ ID No. 34.
- 15 8. Use of a lipid acyltransferase to produce one or more of a carbohydrate ester, a protein ester, a protein subunit ester, or a hydroxy acid ester by catalysis of one or both of alcoholysis or transesterification in an admixture of an acyl donor, an acyl acceptor and water, which admixture comprises 5-98% water, wherein
- 20 said acyl donor is a lipid substrate selected from one or more of the group consisting of a phospholipid, a lysophospholipid, a triacylglyceride, a diglyceride, a glycolipid or a lysoglycolipid and said acyl acceptor is selected from one or more of the group consisting of a carbohydrate, a protein, a protein subunit, or a hydroxy acid.
- 25 9. Use according to claim 8 wherein the lipid acyltransferase is immobilised.
- 30 10. Use according to claim 8 wherein the carbohydrate ester, protein ester, protein subunit ester or a hydroxy acid ester is purified.

11. Use according to any one of claims 8-10 wherein the lipid acyltransferase is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.
- 5 12. Use according to any one of claims 8-11 wherein the lipid acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.
- 10 13. Use according to any one of claims 8-12 wherein the lipid acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas* and *Candida*.
- 15 14. Use according to any one of claims 8-13 wherein the lipid acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix) the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, or an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32 or SEQ ID No. 34.
- 20
- 25
- 30
15. A carbohydrate ester produced by a method according to any one of claims 1-7.
16. A protein ester produced by a method according to any one of claims 1-7.

17. A protein subunit ester produced by a method according to any one of claims 1-7.
18. A hydroxy acid ester produced by a method according to any one of claims 1-7.
19. An immobilised lipid acyltransferase enzyme.
- 5 20. An immobilised lipid acyltransferase according to claim 19 wherein the lipid acyltransferase is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.
- 10 21. An immobilised lipid acyltransferase according to claim 19 or claim 20 wherein the lipid acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.
- 15 22. An immobilised lipid acyltransferase according to any one of claims 19-21 wherein the lipid acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*,
20 *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium*, *Ralstonia*, *Xanthomonas* and *Candida*.
23. An immobilised lipid acyltransferase according to any one of claims 19-22 wherein the lipid acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the
25 amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix)
30 the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino

acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, or an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32 or SEQ ID No. 34.

Figure 1

SEQ ID No. 1

```

1 ivafGD$1Td geayygdsgd ggwgagladr Ltallrlrar prgvdvfnrg isGrtsdGr1
61 ivDalvallF laqslglpnL pPYLsgdflr GANFAsagAt Ilptsgpfli QvqFkdksq
121 vlelrqalgl lqellrlpv ldakspdlvt imiGtNDlit saffgpkste sdrnsvpef
181 kdnlrqlikr Lrsnngarii vlitlvilnl gplGC1Plkl alalassknv dasgclerln
241 eavadfneal relaiskled qlrkdglp dv kgadvpyvDl ysifqdldgi qnpsayvyGF
301 ettkaCCGyG gryNynrvCG naglcnvtaK aCnpssylls flfwDgfHps ekGykavAea
361 1

```

Figure 2

SEQ ID No. 2

```

1 mkkwfvcllg lvaltvqaad srpafsrivm fgdsldstgk myskmrgylp ssppyyegrf
61 sngpvwleql tnefpgltia neaeggptav aynkiwnpk yqvinnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakeill fnlpldgqnp
181 sarsqkvvea ashvsayhnq lllnlarqla ptgmvlkfei dkqfaemlrd pqnfglsdqr
241 nacyggsyvw kpfasrsast dsqlsafnpq erlaiagnpl laqavaspma arsastlnce
301 gkmfwdqvhp ttvvhhaalse paatfiesqy eflah

```

Figure 3

SEQ ID No. 3

```

1 mkkwfvcllg lialtvqaad trpafsrivm fgdsldstgk myskmrgylp ssppyyegrf
61 anllclrsta nyphviadt garltdvtcg aaqtadfta qypgvapqld algtgtdlvt
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakqill fnlpldgqnp
181 sarsqkvvea vshvsayhnk lllnlarqla ptgmvlkfei dkqfaemlrd pqnfglsdve
241 npcydggysv kpfatrsrst drqlsafspq erlaiagnpl laqavaspma rrsasplnce
301 gkmfwdqvhp ttvvhhaalse raatfietqy eflahg

```

Figure 4

SEQ ID No. 4

```

1 mpkpalrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
61 anllclrsta nyphviadt garltdvtcg aaqtadfta qypgvapqld algtgtdlvt
121 ltiggnndst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgywitpat adpscflklp laagdvpplr aiqahlnlav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnaig errmaehtmd vlgld

```

Figure 5

SEQ ID No. 5

```
1 mpkpalrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
61 anlcllrsta nyphviadtt garltdvtcg aaqtadftra qypgvapqld algtgtldlt
121 ltiggnndst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgypwitpat adpscflklp laagdvpplr aiqahldav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnalg errmaehtmd vlglid
```

Figure 6

SEQ ID No. 6

```
1 mdyekfllfg dsitefafnt rpiedgkdqy algaalvney trkmdilqrg fkgytsrwal
61 kilpeilkhe snivmatifl gandacsagp qsvplpefid nirqmvslmk syhirpiiig
121 pglvdrekwe kekseeialg yfrtnenfai ysdalaklan eekvpfvaln kafqgegga
181 wqqlitdqlh fsgkgykifh dellkvietf ypqyhpknmq yklkdwravl ddgsnims
```

Figure 7

Alignment of pfam00657.6 consensus sequence with P10480

```

*->ivafGDSLtdg.....eayygdsgggwgagladrL
iv+fGDSL+d+++ ++ ++ ++++++ ++s+g w ++l + +
P10480 28 IVMFGDSLSDTgkmyskmrgylpssppYYEGRFSNGPVWLEQLTNEF 74

tall..rlrarprgvdvfnrgisGrtsdGrlivDalvallFlaqlglpn
+ l + ++++++ +n+ +
P10480 75 PGLTIANEAEGGPTAVAYNKISWNP----- 100

lpPYLsgdflrGANFAsagAtIlptsgpfliQvqFkdfksqvlelrqalg
++ ++
P10480 101 -----YQVINN 106

llqellrllpvlidakspdlvtimiGtNDlitsaffgpkstesdrnvsupe
l++e+ ++l +++ k+ dlv++++G+ND+ ++ ++ +++++
P10480 107 LDYEVTQFLQKDSFKPDDLVLWVGANDY-----LAYGWNTQDAKR 148

fkdnlrqlikrLrsnngariivlitlvilnlglpGClPlklalalasskn
++d +++++r+ nga+ +++++nl+ lG+ P+
P10480 149 VRDAISDAANRMV-LNGAK-----EILLFNLPDLGQNPS----- 181

vdasgclerlneavadfnealrelaiskledqlrkdglpdkvadpvyvD
+++ +e + ++a++n++l +la +ql+++g+++++d +++
P10480 182 ARSQKVVEAASHVSAYHNQLLLNLA-----RQLAPTGMVKLFEDKQFAE 226

lysifqlddgiqnpsayv.y....GFe.ttkaCCGyGgr.yNyn.rv.CG
+ +q+++ + + +a+++++ +++ ++++++ +N+++r+ ++
P10480 227 MLRDPQNFGLSDQRNACYgGsyvwKPFaSRASSTDSQLSaFNPQeRLaIA 276

nag.l.c.nvtakaC.npssyll.sflfwDgfHpsekGykavAeal<-*
+++ l + +++++a+ +s+ +++++fwD++Hp+ ++a+ e
P10480 277 GNPLLaQaVASPMARrSASTLNCEGKMFWDQVHPTTVVHAALSEPA 322

```

Alignment of pfam00657.6 consensus sequence with AAG09804

```

*->ivafGDSLtdg.....eayygdsgggwgagladrL
iv+fGDSL+d+++ ++ ++ ++++++ ++s+g w ++l + +
AAG09804 28 IVMFGDSLSDTgkmyskmrgylpssppYYEGRFSNGPVWLEQLTKQF 74

tallrlrarprgvdvfnrgisGrtsdGrlivDalvallFlaqlglpnLp
+g+++ n + +G+t
AAG09804 75 -----PGLTIANEAEGGAT----- 88

PYLsgdflrGANFAsagAtIlptsgpfliQvqFkdfksqvlelrqa....
++++ + ++++ +
AAG09804 89 -----AVAYNKISWNPkyq 102

..lgllqellrllpvlidakspdlvtimiGtNDlitsaffgpkstesdrnv
++l++e+ ++l +++ k+ dlv++++G+ND+ ++ ++ ++
AAG09804 103 vYNNLDYEVTQFLQKDSFKPDDLVLWVGANDY-----LAYGWNTQEQ 144

svpefkdnlrqlikrLrsnngariivlitlvilnlglpGClPlklalala
+++++d +++++r+ nga+ +++++nl+ lG+ P+
AAG09804 145 DAKRVRDAISDAANRMV-LNGAK-----QILLFNLPDLGQNPS----- 181

ssknvdasgclerlneavadfnealrelaiskledqlrkdglpdkvadp
+++ +e + ++a++n++l +la +ql+++g+++++d
AAG09804 182 ----ARSKVVEAVSHVSAYHNKLLNLA-----RQLAPTGMVKLFEDK 222

pyvDlysifqlddgiqnpsayv.y....GFe.ttkaCCGyGgr.yNyn.r
+++++ +q+++ + ++ ++++++ +++ t++ +++ ++ + +++r
AAG09804 223 QFAEMLRDPQNFGLSDVENPCYdGgyvwKPFaTRSVSTDRQLSaFSPQeR 272

v.CGnag.l.c.nvtakaC.npssyll.sflfwDgfHpsekGykavAeal
+ +++++ l + +++++a+ +s +++++fwD++Hp+ ++a+ e+
AAG09804 273 LaIAGNP1LaQaVASPMARrSASPLNCEGKMFWDQVHPTTVVHAALSERA 322

```

<-*

AAG09804

Alignment of pfam00657.6 consensus sequence with NP_631558

```

*->ivafGDSLtdgeayygdsdgggwgagladrltallrlrarprgvdvf
+va+GDS ++g          +g + +++L      + + + ++ +
NP_631558  42  YVALGDSYSAG-----SGVLPVDPANL----LCLRSTANYPHV 75

nrgisGrtsdGrliV.D.a.l.vallFlaqlslglpnLpPYLsgdflrGANF
+ ++G++          D + + +
NP_631558  76  IADTTGAR-----LTDvTcGaAQ-----93

AsagAtIlptsgpfliQvqFkdfksqvlelrqalglgellrllpvldak
+++          ++ + ++ +++
NP_631558  94  -----TADFTRAQYPGVAPQLDALGT 114

spdlvtimiGtNDL.....itsaffgpkstesdrnsvvp
+ dlvt+ iG+ND ++ + + ++ + ++ + +k ++ + +++
NP_631558  115 GTDLVTLTIGGNDNstfinaitacgtagvISGGKGSCKDRHGTSFDDEI 164

efkdn..lrqlikrLrs.nngariivlitlvilnlg.....plG
e +++ l++++ +r+++ +ar+ +l +++++ +++ + + G
NP_631558  165 EANTYpaLKEALLGVRARAPHARVAALGYPWITPATadpscfklklplAAG 214

ClPlklalalassknvdasgclerlneavadfnealrelaiskledqlrk
P+          l+ ++a n a+r a
NP_631558  215 DVPY-----LRAIQAHLNDAVRRAA-----234

dglpdvkgadvpyvDlysifqldgignpsayvyGFettkaCCGyGgryn
++ + +yvD+ ++
NP_631558  235 -----EETGATYVDFSGVSDG-----250

ynrvCGnaglcnavtakaC.npssyll.sflfwDgf...HpsekGykavAe
++aC+ p +++ + lf + + + Hp++ G +++Ae
NP_631558  251 -----HDACeAPGTRWIEPLLFHGSLvpvHPNALGERRMAE 286

al<-*
+
NP_631558  287 HT      288

```

Alignment of pfam00657.6 consensus sequence with CAC42140

```

*->ivafGDSLtdgeayygdsdgggwgagladrltallrlrarprgvdvf
+va+GDS ++g          +g + +++L      + + + ++ +
CAC42140  42  YVALGDSYSAG-----SGVLPVDPANL----LCLRSTANYPHV 75

nrgisGrtsdGrliV.D.a.l.vallFlaqlslglpnLpPYLsgdflrGANF
+ ++G++          D + + +
CAC42140  76  IADTTGAR-----LTDvTcGaAQ-----93

AsagAtIlptsgpfliQvqFkdfksqvlelrqalglgellrllpvldak
+++          ++ + ++ +++
CAC42140  94  -----TADFTRAQYPGVAPQLDALGT 114

spdlvtimiGtNDL.....itsaffgpkstesdrnsvvp
+ dlvt+ iG+ND ++ + + ++ + ++ + +k ++ + +++
CAC42140  115 GTDLVTLTIGGNDNstfinaitacgtagvISGGKGSCKDRHGTSFDDEI 164

efkdn..lrqlikrLrs.nngariivlitlvilnlg.....plG
e +++ l++++ +r+++ +ar+ +l +++++ +++ + + G
CAC42140  165 EANTYpaLKEALLGVRARAPHARVAALGYPWITPATadpscfklklplAAG 214

ClPlklalalassknvdasgclerlneavadfnealrelaiskledqlrk
P+          l+ ++a n a+r a
CAC42140  215 DVPY-----LRAIQAHLNDAVRRAA-----234

dglpdvkgadvpyvDlysifqldgignpsayvyGFettkaCCGyGgryn
++ + +yvD+ ++
CAC42140  235 -----EETGATYVDFSGVSDG-----250

ynrvCGnaglcnavtakaC.npssyll.sflfwDgf...HpsekGykavAe
++aC+ p +++ + lf + + + Hp++ G +++Ae
CAC42140  251 -----HDACeAPGTRWIEPLLFHGSLvpvHPNALGERRMAE 286

```

al<-*
+
CAC42140 287 HT 288

Alignment of pfam00657.6 consensus sequence with P41734

```

*->ivafGDSlTdg...eayygdsdgggwgagladrLtallrlrarprg
++fGDS+T+ .+++ + + d+ ga+l + + +r+
P41734 6 FLLFGDSITEFafntRPIEDGKDQYALGAALVNEY-----TRK 43

vdvfnrgisGrtsdGrliVdalvallFlaqlslglpnlPpPYLsgdfllrGAN
+d+ rg++G+t
P41734 44 MDILQRGFKGYT----- 55

FASagAtIlptsgpfliQvqFkdfksqvlrlrqalglqellrllpvlida
+r+al++l+e+l+ +
P41734 56 -----SRWALKILPEILKH-----E 70

kspdlvtimiGtNDlitsaffgpkstesdrnsvsvefkdnrlqlikrLrs
+ + ti++G+ND+ ++ +++ v++pef+dn+rq+++++s
P41734 71 SNIVMATIFLGANDA-----CSAGPQSVPLPEFIDNIRQMVSMLKS 111

nngariivlitlviInlgplGCIPklalalassknvdasgclerlneav
++++i++++lv ++ ++ k ++ + + r+ne +
P41734 112 YHIRPIIIGPGLVDREKW-----EKEKSEEIALGYFRTNENF 148

adfnealrelaiskledqlrkdglpdkvadpvyvDlysifqldldgiqnp
a + al +la ++ +vp+v l+++fq+ +g++++
P41734 149 AIYSDALAKLA-----NEEKVPFVALNKAFAQEGGDAWQ 182

sayvyGFettkaCCGyGgryNynrvCGnaglcnavtakaCnpssyllsflf
+
P41734 183 Q-----LL 185

wDgfHpsekGykavAeal<-*
Dg+H+s kGyk+++++l
P41734 186 TDGLHFSGKGYKIFHDEL 203

```

Figure 8

```

A.sal 1  MKKWFVCLLGLIALTVQAADTRPAFSRIVMFGDSTSDTGKMYSKMRGYLPSSPPYYEGRF 60
          +          +
A.hyd 1  MKKWFVCLLGLVALTVOAADSRPAFSRIVMFGDSTSDTGKMYSKMRGYLPSSPPYYEGRF 60

A.sal 61  SNGPVWLEQLTKQFPGLTIANEAEGGATAVAYNKISWNPKYQVINNLDYEVTQFLQKDSF 120
          ++          +
A.hyd 61  SNGPVWLEQLTNEFPGLTIANEAEGGPTAVAYNKISWNPKYQVINNLDYEVTQFLQKDSF 120

A.sal 121 KPDDLVLWVGANDYLAYGWNTQDAKRVRDAISDAANRMVLNGAKQILLFNLPDLGQNP 180
          +
A.hyd 121 KPDDLVLWVGANDYLAYGWNTQDAKRVRDAISDAANRMVLNGAKEILLFNLPDLGQNP 180

A.sal 181 SARSQKVVEAVSHVSAYHNKLLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDVE 240
          +          +
A.hyd 181 SARSQKVVEAASHVSAYHNQLLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDQR 240 ++

A.sal 241 NPCYDGGYVWKPFATRSVSTDROLSAFSPQERLAIAGNPLLAQAVASPMARRSASPLNCE 300
          + ++ +          + + +          +
A.hyd 241 NACYGGSYVWKPFASRSASTDSQLSAFNPQERLAIAGNPLLAQAVASPMARSASTLNCE 300

A.sal 301 GKMFWDQVHPPTTVVHAALSERAAFTIETQYEFLAH 335
          +          +
A.hyd 301 GKMFWDQVHPPTTVVHAALSEPAATFIESQYEFLAH 335

```

Figure 9

```
1  ATGAAAAAAT GGTTCGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA GGCAGCCGAC
61  AGCCGTCCCG CCTTCTCCCG GATCGTGATG TTTGGCGACA GCCTCTCCGA TACCGGCAAG
121 ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCCC CCTACTATGA GGGCCGCTTC
181 TCCAACGGGC CCGTCTGGCT GGAGCAGCTG ACCAACGAGT TCCCGGGCCT GACCATAGCC
241 AACGAGGCGG AAGGCGGACC GACCGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
301 TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCCTGCAAAA AGACAGCTTC
361 AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGCGCCAACG ACTATCTGGC CTATGGCTGG
421 AACACAGAGC AGGATGCCAA GCGGGTGCGC GACGCCATCA GCGATGCGGC CAACCGCATG
481 GTGCTGAACG GCGCCAAGGA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCC
541 TCGGCCCCGA GCCAGAAGGT GGTGAGGCG GCCAGCCATG TCTCCGCCTA CCACAACCAG
601 CTGCTGCTGA ACCTGGCAGC CCAGCTGGCT CCCACCGGCA TGGTGAAGCT GTTCGAGATC
661 GACAAGCAGT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACCAGAGG
721 AACGCCTGCT ACGGTGGCAG CTATGTATGG AAGCCGTTTG CCTCCGCGAG CGCCAGCACC
781 GACAGCCAGC TCTCCGCCTT CAACCCGCGAG GAGCGCCTCG CCATCGCCGG CAACCCGCTG
841 CTGGCCCAGG CCGTCGCCAG CCCCATGGCT GCCCGCAGCG CCAGCACCCT CAACTGTGAG
901 GGCAAGATGT TCTGGGATCA GGTCCACCCC ACCACTGTCG TGCACGCCGC CCTGAGCGAG
961 CCCGCCGCCA CCTTCATCGA GAGCCAGTAC GAGTTCCTCG CCCAC
```

Figure 10

```

1  ATGAAAAAAT GGTTCGTTTG TTTATTGGGG TTGATCGCGC TGACAGTTCA GGCAGCCGAC
61  ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA GCCTCTCCGA TACCGGCAAA
121 ATGTACAGCA AGATCGCGCG TTACCTCCCC TCCAGCCCGC CCTACTATGA GGGCCGTTTC
181 TCCAACGGAC CCGTCTGGCT GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC
241 AACGAAGCGG AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
301 TATCAGGTCT ACAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA AGACAGCTTC
361 AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG ACTATCTGGC ATATGGCTGG
421 AATACGGAGC AGGATGCCAA GCGAGTTCGC GATGCCATCA GCGATGCGGC CAACCGCATG
481 GTACTGAACG GTGCCAAGCA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG
541 TCAGCCCGCA GTCAGAAGGT GGTGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACAAG
601 CTGCTGCTGA ACCTGGCAGC CCAGCTGGCC CCCACCGGCA TGGTAAAGCT GTTCGAGATC
661 GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACGTCGAG
721 AACCCTGCT ACGACGGCGG CTATGTGTGG AAGCCGTTTG CCACCCGAG CGTCAGCACC
781 GACCGCCAGC TCTCCGCCTT CAGTCCGCG GAAAGCCTCG CCATCGCCGG CAACCCGCTG
841 CTGGCACAGG CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
901 GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCTG TGCACGCAGC CCTGAGCGAG
961 CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG CCCACGGATG A

```

Figure 11

```
1  ATGCCGAAGC CTGCCCTTCG CCGTGTCATG ACCGCGACAG TCGCCGCCGT CGGCACGCTC
61  GCCCTCGGCC TCACCGACGC CACCGCCAC GCCGCGCCG CCCAGGCCAC TCCGACCCTG
121 GACTACGTCG CCTTCGGCGA CAGCTACAGC GCCGGCTCCG GCGTCCTGCC CGTCGACCCC
181 GCCAACCTGC TCTGTCTGCG CTCGACGGCC AACTACCCCC ACGTCATCGC GGACACGACG
241 GCGCCCCGCC TCACGGACGT CACCTGCGGC GCCGCGCAGA CCGCCGACTT CACGCGGGCC
301 CAGTACCCGG GCGTCGCACC CCAGTTGGAC GCGCTCGGCA CCGGCACGGA CCTGGTCACG
361 CTCACCATCG GCGGCAACGA CAACAGCACC TTCATCAACG CCATCACGGC CTGCGGCACG
421 GCGGGTGTCC TCAGCGGCGG CAAGGGCAGC CCCTGCAAGG ACAGGCACGG CACCTCCTTC
481 GACGACGAGA TCGAGGCCAA CACGTACCCC GCGCTCAAGG AGGCGCTGCT CGGCGTCCGC
541 GCCAGGGGTC CCCACGCCAG GGTGGCGGCT CTCGGCTACC CGTGGATCAC CCCGGCCACC
601 GCCAGCCCGT CCTGCTTCCT GAAGTCCCC CTCGCCGCCG GTGACGTGCC CTACCTGCGG
661 GCCATCCAGG CACACCTCAA CGACGCGGTC CGGCGGGCCG CCGAGGAGAC CGGAGCCACC
721 TACGTGGACT TCTCCGGGGT GTCCGACGGC CACGACGCCT GCGAGGCCCC CGGCACCCGC
781 TGGATCGAAC CGCTGCTCTT CGGGCACAGC CTCGTTCCCC TCCACCCCAA CGCCCTGGGC
841 GAGCGGCACA TGGCCGAGCA CACGATGGAC GTCTCGGCC TGGACTGA
```

Figure 12

```

1   TCAGTCCAGG CCGAGGACGT CCATCGTGTG CTCGGCCATG CGCCGCTCGC CCAGGGCGTT
61  GGGGTGGACG GGAACGAGGC TGTGCCCAGG GAGCAGCGGT TCGATCCAGC GGGTGCCGGG
121 GGCTTCGCAG GCGTCGTGGC CGTCGGACAC CCCGAGAGAG TCCACGTAGG TGGCTCCGGT
181 CTCCTCGGCG GCCCGCCGGA CCGCGTCGTT GAGGTGTGCC TGGATGGCCC GCAGGTAGGG
241 CACGTCACCG GCGGCGAGGG GGAGCTTCAG GAAGCAGGAC GGGTCGGCGG TGGCCGGGGT
301 GATCCACGGG TAGCCGAGAG CCGCCACCC T GCGTGGGGA GCCCTGGCGC GGACGCCGAG
361 CAGCGCCTCC TTGAGCGCGG GGTACGTGTT GGCTCGATC TCGTCGTCGA AGGAGGTGCC
421 GTCCCTGTCC TTGCAGGGGC TGCCCTTGCC GCGCTGAGG ACACCCGCCG TGCCGCAGGC
481 CGTGATGGCG TTGATGAAGG TGCTGTTGTC GTTGCCGCCG ATGGTGAGCG TGACCAGGTC
541 CGTGCCGGTG CCGAGCGCGT CCAACTGGGG TGCGACGCC GGGTACTGGG CCCGCGTGAA
601 GTCGCGGGTC TGC CGCGCGC CGCAGGTGAC GTCCGTGAGG CCGGC GCCCG TCGTGTCCGC
661 GATGACGTGG GGGTAGTTGG CCGTCGAGCG CAGACAGAGC AGGTTGGCGG GGTGACGGG
721 CAGGACGCCG GAGCCGGCGC TGTAGCTGTC GCCGAGGGCG ACGTAGTCCA GGGTCGGAGT
781 GGCTGGGCG GCGCGGCGT GGGCGGTGGC GTCGGTGAGG CCGAGGGCGA GCGTGCCGAC
841 GCGGCGGACT GTCGCGGTCA TGACACGGCG AAGGGCAGGC TTCGGCAT

```

Figure 13

```
1  ATGGATTACG AGAAGTTTCT GTTATTTGGG GATTCCATTA CTGAATTTC TTTAATACT
61  AGGCCCATTC AAGATGGCAA AGATCAGTAT GCTCTTGGAG CCGCATTAGT CAACGAATAT
121 ACGAGAAAAA TGGATATTCT TCAAAGAGGG TTCAAAGGGT ACACTTCTAG ATGGGCGTTG
181 AAAATACTTC CTGAGATTTT AAAGCATGAA TCCAATATTG TCATGGCCAC AATATTTTGT
241 GGTGCCAACG ATGCATGCTC AGCAGGTCCC CAAAGTGTC CCCTCCCCGA ATTTATCGAT
301 AATATTCGTC AAATGGTATC TTTGATGAAG TCTTACCATA TCCGTCCTAT TATAATAGGA
361 CCGGGGCTAG TAGATAGAGA GAAGTGGGAA AAAGAAAAAT CTGAAGAAAT AGCTCTCGGA
421 TACTTCCGTA CCAACGAGAA CTTTGCCATT TATTCGATG CCTTAGCAAA ACTAGCCAAT
481 GAGGAAAAAG TTCCCTTCGT GGCCTTGAAT AAGGCGTTTC AACAGGAAGG TGGTGATGCT
541 TGGCAACAAC TGCTAACAGA TGGACTGCAC TTTCCCGAA AAGGGTACAA AATTTTTCAT
601 GACGAATTAT TGAAGTCAT TGAGACATTC TACCCCAAT ATCATCCAA AACATGCAG
661 TACAACTGA AAGATTGGAG AGATGTGCTA GATGATGGAT CTAACATAAT GTCTTGA
```


Figure 14

(SEQ ID No. 12)

10	20	30	40	50	60
MNLRQWMGAA	TAALALGLAA	CGGGGTDQSG	NPNVAKVQRM	VVFGDSLSDI	GYTFVAQAV
70	80	90	100	110	120
GGGKFTTNPG	PIWAETVAAQ	LGVTLTPAVM	GYATSVQNCP	KAGCFDYAQQ	GSRVTDPNGI
130	140	150	160	170	180
GHNGGAGALT	YPVQQQLANF	YAASNNTFNG	NNDVVFVLAG	SNDIFFWTTA	AATSGSGVTP
190	200	210	220	230	240
AIATAQVQQA	ATDLVGIVKD	MIAKGATQVY	VFNLPDSSLT	PDGVASGTTG	QALLHALVGT
250	260	270	280	290	300
FNTTLQSGLA	GTSARIIDFN	AQLTAAIQNG	ASFGFANTSA	RACDATKINA	LVPSAGGSSL
310	320	330	340		
FCSANTLVAS	GADQSYLFAD	GVHPTTAGHR	LIASNVLARL	LADNVAH	

Figure 15

(SEQ ID No. 13)

atgaacctgc gtcaatggat gggcgccgcc acggctgccc ttgccttggg cttggccgcg	60
tcggggggcg gtgggaccga ccagagcggc aatcccaatg tcgccaaggt gcagcgcag	120
gtggtgttcg gcgacagcct gagcgatata ggcacctaca ccccgctcgc gcaggcggtg	180
ggcgccggca agttcaccac caaccgggc ccgatctggg ccgagaccgt ggccgcgcaa	240
ctgggctga cgctcacgcc ggcggtgatg ggctacgcca cctccgtgca gaattgccc	300
aaggccggct gcttcgacta tgcgcagggc ggctcgcgcg tgaccgatcc gaacggcatc	360
ggccacaacg gcggcgcggg ggcgctgacc taccgggttc agcagcagct cgccaacttc	420
tacgcggcca gcaacaacac attcaacggc aataacgatg tcgtcttcgt gctggccggc	480
agcaacgaca ttttcttctg gacctctgcg gcggcctacca gcggctccgg cgtgacgcc	540
gccattgcca cggcccaggt gcagcaggcc gcgacggacc tggtcggcta tgtcaaggac	600
atgatcgcca aggggtcgac gcaggtctac gtgttcaacc tgcccgacag cagcctgacg	660
ccggacggcg tggcaagcgg cacgaccggc caggcgtgc tgcacgcgct ggtgggcacg	720
ttcaacacga cgctgcaaag cgggctggcc ggcacctcgg cgcgcacatc cgacttcaac	780
gcacaactga ccgcggcgat ccagaatggc gcctcgttcg gcttcgcca caccagcgcc	840
cgggcctgcg acgccacca gatcaatgcc ctggtgccga gcgccggcg cagctcgctg	900
ttctgctcgg ccaacacgct ggtggcttcc ggtgcggacc agagctacct gttcgccgac	960
ggcgtgcacc cgaccacggc cgcccatcgc ctgatcgcca gcaacgtgct ggcgcgcctg	1020
ctggcgata acgtcgcgca ctga	1044

Figure 16 (SEQ ID No. 20)

```
1 migsyvavgd sftegvgdpg pdgafvgwad rlavlladrr pegdftytnl avrgrlldqi
61 vaegvprvvg lapdlvsfaa ggndiirpgt dpdevaerfe lavaalataa gtvltvtgfd
121 trgvvpvlkhl rgkياتyngh vraiadrygc pvlldlslrs vqdrrawdada rhlhspeght
181 rvalraggal glrvpadpdq pwpplpprgt ldvrrddvhw areylvpwig rrlrgessgd
241 hvtakgtlsp daiktriaav a
```

Figure 17 (SEQ ID No. 21)

```
1 gtgacgsggt cgtacgtggc ggtgggggac agcttcaccg agggcgctcg cgaccccggc
61 cccgacgggg cgttcgtcgg ctgggcccgc cggctcgccg tactgctcgc ggaccggcgc
121 cccgagggcg acttcacgta cacgaacctc gccgtgcgcg gcaggctcct cgaccagatc
181 gtggcggaac aggtcccgcg ggtcgtcgga ctgcgcgccg acctcgtctc gttcgcggcg
241 ggcggaacg acatcatccg gcccggcacc gatcccgacg aggtcgccga gcggttcgag
301 ctggcggtgg ccgcgctgac cgcgcgggcc ggaaccgtcc tggtagaccac cgggttcgac
361 acccgggggg tgccgctcct caagcacctg cgcggaaga tcgccacgta caacgggcac
421 gtccgcgcca tcgccgaccg ctacggctgc ccggtgctcg acctgtggtc gctgcggagc
481 gtccaggacc gcaggcgctg ggacggccgc cggctgcacc tgtcgccgga ggggcacacc
541 cgggtggcgc tgcgcgcggg gcaggccctg ggctgcgcg tcccgccga ccctgaccag
601 ccctggccgc ccctgcgcgc gcgcggcacg ctgcagctcc ggcgcgacga cgtgactgg
661 gcgcgcgagt acctggtgcc gtggatcggg cgccggctgc ggggcgagtc gtcgggcgac
721 cacgtgacgg ccaaggggac gctgtcgccg gacgccatca agacgcggat cgccgcggtg
781 gcctga
```

Figure 18
(SEQ ID No. 22)

```

1  mqtnpaytsl vavgdsfteg msdlldgtsy rgwadllatr maarspgfry anlavrgkli
61  gqivdeqv dv aaamgadvit lvggln dtlr pkcdmarvrd lltqaverla phceqlvlmr
121 spgrqgppvle rfrprmealf aviddlagr h gavvvdlyga qsladprmw d vdrhlhtaeg
181 hrrvaeavwq slghepedpe whapipatpp pgwvtrrtad vrfarqhllp wigxrltgrs
241 sgdglpakrp dlipyedpar

```

Figure 19 (SEQ ID No. 23)

```

1  atgcagacga accccgcgta caccagtctc gtcgccgtcg gcgactcctt caccgagggc
61  atgtcggacc tgctgcccga cggctcctac cgtggctggg cgcacctcct cgccaccg
121 atggcggccc gctcccccg cttccggtac gccaacctgg cgggtgcgcg gaagctgac
181 ggacagatcg tcgacgagca ggtggacgtg gccgccgcca tgggagccga cgtgatcac
241 ctggtcggcg ggctcaacga cacgtgcgg cccaagtgcg acatggcccg ggtgcgggac
301 ctgctgaccc aggcctgga acggtcgc cgcactgcg agcagctggt gctgatgcg
361 agtcccggtc gccaggggtc ggtgctggag cgcttcggc cccgcatgga ggccctgtt
421 gccgtgatcg acgacctggc cgggcggcac ggcgcgtgg tcgtcgacct gtacggggc
481 cagtcgctgg ccgacctcg gatgtgggac gtggaccggc tgcacctgac cgccgagggc
541 caccgccggg tcgcgaggc ggtgtggcag tcgctcggc acgagccga ggaccccgag
601 tggcacgcgc cgatccggc gacgccggc ccgggggtgg tgacgcgcag gaccgcggac
661 gtccgggttcg cccggcagca cctgctgcc tggataggcc gcaggctgac cgggcgctcg
721 tccggggacg gcctgccggc caagcgccg gacctgctg cctacagga ccccgcacg
781 tga

```

Figure 20 (SEQ ID No. 24)

```

1 mtrgrdggag apptkhrall aaivtlivai saaiyagasa ddgsrdhalq aggrlprgda
61 apastgawvg awatapaaae pgtettglag rsvrnvvhts vggtagariti snlyggsplt
121 vthasialaa gpdtaaaiaa tmrrltfggs arviipaggg vmsdtarlai pyganvlvtt
181 yspipsgpvt yhpqarqtsy ladgdrtadv tavayttptp ywryltaldv lsheadgtvv
241 afgdsitdga rsqsdanhrw tdvlaarlhe aagdgdrdtp ysvvnegisg nrlltsrpgr
301 padnpsglr fqrdrvleritn vkavvvvlgv ndvlinspela drdailtglr tlvdraharg
361 lrvvgatitp fggygygytea retmrqevne eirsgrvfdt vvdfdkalrd pydprmrds
421 ydsqgdhlpq dkgayarmgav idlaalkgaa pvka

```

Figure 21 (SEQ ID No. 25)

```

1 atgacccggg gtcgtgacgg ggggtgcgggg gcgccccca ccaagcaccg tgccctgctc
61 gcggcgatcg tcaccctgat agtggcgatc tccgcggcca tatacgccgg agcgtcccg
121 gacgacggca gcagggacca cgcgctgcag gccggaggcc gtctcccacg aggagacggc
181 gcccccggt ccaccgggtgc ctgggtgggc gcctgggcca ccgcaccggc cgcggccgag
241 ccgggacccg agacgaccgg cctggcgggc cgctccgtgc gcaacgtcgt gcacacctcg
301 gtcggcgcca ccggcgcgcg gatcacctc tcgaacctgt acgggcagtc gccgtgacc
361 gtcacacacg cctcgatcgc cctggccgcc gggcccgaca ccgcccgcgc gatcgccgac
421 accatgcgcc ggctcacctt cggcggcagc gcccggtga tcatcccgcg gggcgccag
481 gtgatgagcg acaccgccc cctcgccatc ccctacgggg cgaacgtcct ggtcaccacg
541 tactcccca tcccgctccg gccggtgacc taccatccgc aggccggca gaccagctac
601 ctggccgacg gcgaccgcac ggcggacgtc accgcccgtc cgtacaccac cccacgccc
661 tactggcgct acctgaccgc cctcgacgtg ctgagccacg aggcggacgg cacggtcgtg
721 gcgttcggcg actccatcac cgacggcgcc cgctcgaga gcgacgcaa ccaccgctgg
781 accgacgtcc tgcgcgcacg cctgcacgag gcggcgggcg acggccggga cagccccgc
841 tacagcgteg tcaacgaggg catcagcggc aaccggctcc tgaccagcag gccggggcg
901 ccggccgaca acccgagcgg actgagccgg ttccagcggg acgtgctgga acgaccaa
961 gtcaaggccg tcgtcgctgt cctcggcgtc aacgacgtcc tgaacagccc ggaactcgcc
1021 gaccgcgacg ccatcctgac cggcctgcgc accctcgteg accggggcga cggccggga
1081 ctgcgggteg tcggcgccac gatcacgccc ttccggcggt acggcggtc caccgaggcc
1141 cgcgagacga tgcggcagga ggtcaacgag gagatccgct ccggccgggt cttcgacacg
1201 gtcgtcgact tcgacaaggc cctgcgcgac ccgtacgacc cgcgcggat gcgtccgac
1261 tacgacagcg gcgaccacct gaccccggc gacaaggggt acgcgcgcat gggcgcggtc
1321 atcgacctgg ccgcgctgaa gggcgcgggc ccggtcaagg cgtag

```

Figure 22 (SEQ ID No. 26)

```

1 mtsmsrarva rriaagaayg gggiglagaa avglvvaevq larrivgvgt ptrvpnaqgl
61 yggtlptagd pplrlmmlgd staaggvhr agqtpgalla sglaavaerp vrlgsvaqpg
121 acsddldrv alvlaepdrv pdicvimvga ndvthmpat rsvrhlssav rrlrtagaev
181 vvgtcpdlgt iervrqplrw larrasrqla aaqtigaveq ggrtvslgdl lgpefaqnpr
241 elfgpdnyhp saegyataam avlpsvcaal glwpadeehp dalrregflp varaaaaaas
301 eagtevaam ptgprgpwal lkrirrrrvs eaepsspsgv

```

Figure 23 (SEQ ID No. 27)

```

1 atgacgagca tgtcgagggc gaggggtggcg cggcggtatcg cggccggcgc ggcgtacggc
61 ggcggcgcca tcggcctggc gggagcgggc gcggtcggtc tgggtgggtgc cgaggtgcag
121 ctggccagac gcaggtggg ggtgggcacg ccgaccggg tgccgaacgc gcagggactg
181 tacggcgcca ccctgcccac ggcggcgac cgcgcgtgc ggtgatgat gctggcgac
241 tccacggccg ccgggcagg cgtgcaccg gccgggcaga cgcgggcgc gctgctggcg
301 tccgggctcg cggcggtggc ggagcgccg gtgcggctgg ggtcggtcgc ccagccggg
361 gcgtgctcgg acgacctgga ccggcaggtg gcgctggtgc tcgccagacc ggaccgggtg
421 cccgacatct gcgtgatcat ggtcggcgcc aacgacgtca cccaccgat gccggcgacc
481 cgctcggtgc ggcacctgtc ctggcggtg cggcggtgc gcacggccg tcggaggtg
541 gtggtcgcca cctgtccgga cctgggcacg atcgagcggg tcggcagacc gctgcgtgg
601 ctggcccgcc gggcctcacg gcagctcgg gcggcacaga ccacggcgc cgtcgagcag
661 ggcggcgcca cgggtgctcg gggcgacctg ctgggtccgg agttcgcgca gaaccccg
721 gagctcttcg gcccgacaa ctaccacccc tcccgagag ggtacgccac ggcgcgatg
781 gcggtactgc cctcgggtgt cgccgcgtc ggcctgtggc cggccgacga ggagacccc
841 gacgcgtcgc gccgcagggt cttcctgcc gtggcgcgcg cggcggcgga ggcggcgctc
901 gaggcgggta cggaggtcgc cgcggccatg cctacggggc ctcgggggcc ctggggcgtg
961 ctgaagcgcc ggagacggcg tcgggtgtcg gagcggaac cgtccagccc gtccggcgtt
1021 tga

```

Figure 24 (SEQ ID No. 28)

```

1 mgrgtdqrtr ygrrrarval aaltaavlgv gvagcdsvgg dspapsgsps ktrrtapawd
61 tspasvaavg dsitrgfdac avlsdcpevs watgssakvd slavrllgka daaehswnya
121 vtgarmadlt aqvtraagre pelvavmaga ndacrsttsa mtpvadfrac feeamatlrk
181 klpkavvyvs sipdlkrlws qgrtnplgkq vwklglcpms lgdadslds atlrntvrd
241 rvadynevlr evcakdrxcr sddgavhefr fgtdqlshwd wfhpssvdgga rlaeiayrav
301 taknp

```

Figure 25 (SEQ ID No. 29)

```

1 atgggtcgag ggacggacca gcggacgcgg tacggccgctc gccgggcgcg tgctcgctc
61 gccgccctga ccgccgccgt cctgggctgt ggctggcggt gctgcgactc cgtgggcggtc
121 gactcaccgg ctccctccgg cagcccgctc aagcggacga ggacggcgcc cgcctgggac
181 accagcccgg cgtccgtcgc cgcctgggac gactccatca cgcgcggtt cgacgcctgt
241 gcggtgctgt cggactgccc ggaggtgtcg tgggcgaccg gcagcagcgc gaaggtcgac
301 tcgctggccg tacggctgct ggggaaggcg gacgcggccg agcacagctg gaactacggc
361 gtcaccgggg ccgggatggc ggacctgacc gctcaggtga cgcgggcggt gcagcgcgag
421 ccggagctgg tggcggtgat ggccggggcg aacgacgcgt gccggtccac gacctcggtc
481 atgacgccgg tggcgactt ccgggcgag ttcgaggagg cgatggccac cctgcgcaag
541 aagctcccca aggcgcaggt gtacgtgtcg agcatcccgg acctcaagcg gctctgggtc
601 cagggccgca ccaaccgct gggcaagcag gtgtggaagc tcggcctgtg ccgctcgatg
661 ctgggcgacg cggactccct ggactcggcg gcgaccctgc ggcgcaacac ggtgcgcgac
721 cgggtggcgg actacaacga ggtgctgcgg gaggtctgcy cgaaggaccg gcggtgccgc
781 agcgacgacg gcgcggtgca cgagttccgg ttcggcacgg accagttgag ccactgggac
841 tggttccacc cgagtgtgga cggccaggcc cggctggcgg agatcgctca ccgcgcggtc
901 accgcgaaga atccctga

```

Figure 26 (SEQ ID No. 30)

```

1 mrlsrraata salltpala lfgasaavsa priqatdyva lgdsyssvgv agsydsssgs
61 ckrstksypa lwaashtgtr fnftacsgar tgdvlakqlt pvnsqtdlvs itiggnadagf
121 adtmttcnlq gesaclaria karayiqqtl paqlldqvyda idsrapaaqv vvlgyprfyk
181 lggscavglis eksraainaa addinavtak raadhgfafg dvnttfaghe lcsgapwlhs
241 vtlpvensyh ptangqskgy lpvlinsat

```

Figure 27 (SEQ ID No. 31)

```

1 ttcatcaciaa cgatgtcaca acaccggcca tccgggtcat ccctgatcgt gggaatgggt
61 gacaagcctt cccgtgacga aagggtcctg ctacatcaga aatgacagaa atcctgtctca
121 gggaggttcc atgagactgt cccgacgcgc gccacggcg tccgcgctcc tcctcacccc
181 ggcgctcgcg ctcttcggcg cgagcgccgc cgtgtccgcg ccgcgaatcc aggccaccga
241 ctacgtggcc ctgcggcgact cctactcctc gggggctcggc gcgggcagct acgacagcag
301 cagtggctcc tgtaagcgca gcaccaagtc ctaccgggcc ctgtggggcg cctcgcacac
361 cggtagcgcg ttcaacttca ccgcctgttc gggcgcccg acaggagacg tgcggccaa
421 gcagctgacc ccggtcaact ccggcaccga cctggtcagc attaccatcg gcggcaacga
481 cgcgggcttc gccgacacca tgaccacctg caacctccag ggcgagagcg cgtgcctggc
541 gcggatcgcc aaggcgcgcg cctacatcca gcagacgctg cccgcccagc tggaccaggt
601 ctacgacgcc atcgacagcc gggccccgc agcccaggte gtcgtcctgg gctaccgcg
661 cttctacaag ctgggcgcca gctgcgccgt cgggtctctc gagaaagtcg gcgcgccat
721 caacgccgcc gccgacgaca tcaacgccgt caccgccaa gcgcgcgcgc accacggctt
781 cgccttcggg gacgtcaaca cgacctcgc cgggcacgag ctgtgctccg gcgccccctg
841 gctgcacagc gtcacccttc ccgtggagaa ctctaccac cccacggcca acggacagtc
901 caagggctac ctgcccgtcc tgaactccgc cactgatct cgcggctact ccgccccctga
961 cgaagtcccg cccccggcg gggcttcgcc gtaggtgcgc gtaccgccgt cgcctcgcg
1021 gccggtggcc ccgccgtacg tgccgccgcc cccggacgcg gtcggttc

```


Figure 28 (SEQ ID No. 32)

```
1  MKKWFVCLLG LVALTVQAAD SRPAFSRIVM FGDSLSDTGK MYSKMRGYLP
51  SSPPYYEGRF SNGPVWLEQL TKQFPGLTIA NEAEGGATAV AYNKISWNPK
101 YQVINNL DYE VTQFLQKDSF KPDDLVLWV GANDYLAYGW NTEQDAKRVR
151 DAISDAANRM VLNGAKQILL FNLPDLGQNP SARSQKVVEA VSHVSAYHNQ
201 LLLNLARQLA PTGMVKLFEI DKQFAEMLRD PQNFGLSDVE NPCYDGGYVW
251 KPFATRSVST DRQLSAFSPQ ERLAIAGNPL LAQAVASPMA RRSASPLNCE
301 GKMFWQVHP TTVVHAALSE RAATFIANQY EFLAH*
```

Figure 29 (SEQ ID No. 33)

```
1  ATGAAAAAAT GGTTCGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA
   TACTTTTTTA CCAAACACAC AAATAACCCCT AACCAGCGCG ACTGTCAAGT

51  GGCAGCCGAC AGTCGCCCGC CCTTTCCCGG GATCGTGATG TTCGGCGACA
   CCGTCGGCTG TCAGCGGGGC GGAAAAGGGC CTAGCACTAC AAGCCGCTGT

101 GCCTCTCCGA TACCGGCAAA ATGTACAGCA AGATCGCGCG TTACCTCCCC
   CGGAGAGGCT ATGGCCGTTT TACATGTCGT TCTACGCGCC AATGGAGGGG

151 TCCAGCCCGC CTTACTATGA GGGCCGTTC TCCAACGGAC CCGTCTGGCT
   AGGTCGGGCG GGATGATACT CCCGGCAAAG AGGTTGCCTG GGCAGACCGA

201 GGAGCAGCTG ACCAAACAGT TCCCGGGTCT GACCATCGCC AACGAAGCGG
   CCTCGTCGAC TGGTTTGTC AAGGCCCAGA CTGGTAGCGG TTGCTTCGCC

251 AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
   TTCCGCCACG GTGACGGCAC CGAATGTTGT TCTAGAGGAC CTAGGGTTCT

301 TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA
   ATAGTCCAGT AGTTGTTGGA CTGATGCTC CAGTGGGTCA AGAACGTCTT

351 AGACAGCTTC AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG
   TCTGTGGAAG TTCGGCCTGC TAGACCACTA GGAGACCCAG CCACGGTTAC

401 ACTATCTGGC CTATGGCTGG AACACGGAGC AGGATGCCAA GCGGGTTTCG
   TGATAGACCG GATACCGACC TTGTGCCTCG TCCTACGTT CGCCCAAGCG

451 GATGCCATCA GCGATGCCGC CAACCGCATG GACTGAACG GTGCCAAGCA
   CTACGGTAGT CGCTACGCCG GTTGGCGTAC CATGACTTGC CACGGTTCGT

501 GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG TCAGCTCGCA
   CTATGACGAC AAGTTGGACG GCCTAGACCC GGTCTTGGGC AGTCGAGCGT

551 GTCAGAAGGT GGTGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACCAG
   CAGTCTTCCA CCAGCTCCGC CAGTCGGTAC AGAGGCGGAT AGTGTGGTC

601 CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGCA TGGTAAAGCT
   GACGACGACT TGGACCGTGC GGTGACCGG GGTGGCCGT ACCATTTCGA

651 GTTCGAGATC GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT
   CAAGCTCTAG CTGTTCTGTA AACGGCTCTA CGACGCACTA GGCCTCTTGA

701 TCGGCCTGAG CGACGTCGAG AACCCCTGCT ACGACGGCGG CTATGTGTGG
   AGCCGGACTC GCTGCAGCTC TTGGGGACGA TGCTGCCGCC GATACACACC

751 AAGCCGTTTG CCACCCGAG CGTCAGCACC GACCGCCAGC TCTCCGCCTT
   TTCGGCAAAC GGTGGCGTGC GCAGTCGTGG CTGGCGGTGC AGAGGCGGAA

801 CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG CTGGCACAGG
   GTCAGGCGTC CTGCGGAGC GGTAGCGGCC GTTGGGCGAC GACCGTGTCC

851 CCGTTGCCAG TCCTATGGCC CGCCGAGCG CCAGCCCCCT CAACTGTGAG
   GGCAACGGTC AGGATACCGG GCGGCGTCGC GGTGCGGGGA GTTGACACTC

901 GGCAAGATGT TCTGGGATCA GGTACACCG ACCACTGTG TGCACGCAGC
   CCGTCTTACA AGACCCTAGT CCATGTGGGC TGGTGACAGC ACGTGCCTG

951 CCTGAGCGAG CGCGCCGCCA CCTTCATCGC GAACCACTAC GAGTTCCTCG
   GGAATCGCTC GCGCGCGCGT GGAAGTAGCG CTTGGTCATG CTCAAGGAGC

1001 CCCAC TGA
     GGTG ACT
```

Figure 30 (SEQ ID No. 34)

```
1  MKKWFVCLLG LIALTVQAAD TRPAFSRIVM FGDSLSDTGK MYSKMRGYLP
51  SSPPYYEGRF SNGPVWLEQL TKQFPGLTIA NEAEGGATAV AYNKISWNP
101 YQVINNL DYE VTQFLQKDSF KPDDLVLWV GANDYLAYGW NTEQDAKRVR
151 DAISDAANRM VLNCAKQILL FNLPDLGQNP SARSQKVVEA VSHVSAYHNK
201 LLLNLARQLA PTGMVKLFEI DKQFAEMLRD PQNFGLSDVE NPCYDGGYVW
251 KPFATRSVST DRQLSAFSPQ ERLAIAGNPL LAQAVASPM RRSASPLNCE
301 GKMFWQVHP TTVVHAALSE RAATFIETQY EFLAHG*
```

Figure 31 (SEQ ID No. 35)

1 ATGAAAAAAT GGTTCGTTT TTTATTGGGG TTGATCGCGC TGACAGTTCA
TACTTTTTTA CCAAACAAAC AAATAACCCC AACTAGCGCG ACTGTCAAGT

51 GGCAGCCGAC ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA
CCGTCGGCTG TGAGCGGGC GGAAGAGGGC CTAGCACTAC AAGCCGCTGT

101 GCCTCTCCGA TACCGGCAAA ATGTACAGCA AGATCGCGG TTACCTCCCC
CGGAGAGGCT ATGGCCGTTT TACATGTCGT TCTACGCGCC AATGGAGGGG

151 TCCAGCCCGC CCTACTATGA GGGCCGTTTC TCCAACGGAC CCGTCTGGCT
AGGTCGGGCG GGATGATACT CCCGGCAAAG AGGTGCGCTG GGCAGACCGA

201 GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC AACGAAGCGG
CCTCGTCGAC TGGTTCGTCA AGGGCCGAGA CTGGTAGCGG TTGCTTCGCC

251 AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
TTCCGCCACG GTGACGGCAC CGAATGTTGT TCTAGAGGAC CTTAGGGTTC

301 TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCTTGAGAA
ATAGTCCAGT AGTTGTTGGA CCTGATGCTC CAGTGGGTCA AGAACGTCTT

351 AGACAGCTTC AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG
TCTGTGGAAG TTCGGCCTGC TAGACCACTA GGAGACCCAG CCACGGTTAC

401 ACTATCTGGC ATATGGCTGG AATACGGAGC AGGATGCCAA GCGAGTTCGC
TGATAGACCG TATACCGACC TTATGCCTCG TCCTACGGTT CGCTCAAGCG

451 GATGCCATCA GCGATGCGGC CAACCGCATG GACTGAACG GTGCCAAGCA
CTACGGTAGT CGCTACGCCG GTTGGCGTAC CATGACTTGC CACGGTTCGT

501 GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG TCAGCCCGCA
CTATGACGAC AAGTTGGACG GCCTAGACCC GGTCTTGGGC AGTCGGGCGT

551 GTCAGAAGGT GGTGAGGCG GTCAGCCATG TCTCCGCTA TCACAACAAG
CAGTCTTCCA CCAGCTCCGC CAGTCGGTAC AGAGGCGGAT AGTGTGTTC

601 CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGSTAAAGCT
GACGACGACT TGGACCGTGC GGTGACCGG GGTGGCCGT ACCATTTCGA

651 GTTCGAGATC GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT
CAAGCTCTAG CTGTTCTGTA AACGGCTCTA CGACGCACTA GCGCTCTGA

701 TCGGCCTGAG CGACGTCGAG AACCCCTGCT ACGACGGCGG CTATGTGTGG
AGCCGGAATC GCTGCAGCTC TTGGGGACGA TGCTGCCGCC GATACACACC

751 AAGCCGTTTG CCACCCGCGC CGTCAGCACC GACCGCCAGC TCTCCGCTT
TTCGGCAAAC GGTGGGCGTC GCAGTCGTGG CTGGCGGTG AGAGGCGGAA

801 CAGTCCGCGA GAACGCCTCG CCATCGCCGG CAACCCGCTG CTGGCACAGG
GTCAGGCGTC CTGCGGAGC GGTAGCGGCC GTTGGGCGAC GACCGTGTCC

851 CCGTTGCCAG TCCTATGGCC CGCCGAGCG CCAGCCCCCT CAACTGTGAG
GGCAACGGTC AGGATACCGG GCGCGTCTGC GGTGGGGGA GTTGACACTC

901 GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTG TGCACGCAGC
CCGTTCTACA AGACCCTAGT CCATGTGGGC TGGTGACAGC ACGTGCCTCG

951 CCTGAGCGAG CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG
GGACTCGCTC GCGCGCGGT GGAAGTAGCT CTGGGTCATG CTCAGGAGC

1001 CCCACGGATG A
GGGTGCCCTAC T

Figure 32

	1	10	20	30	40	50
	-----+-----+-----+-----+-----					
satA	ADTRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRFSG					
R.sol	QSGNPWYAKVQRMVYFGDSLSDIGT-----YTPYAQAVGGGKFTTNP					
Consensus	...adnraafqRiYnFGDSLSDiGk.....YlPsaqaygeGrFsn..G					
	51	60	70	80	90	100
	-----+-----+-----+-----+-----					
satA	PVNLEQLTKQFPGLTIANEAEGGATAYAYNKISHNPKYQVINNLDEYVTQ					
R.sol	PINAETVAAQL-GVTLTPAYMGYATSVQNCCKAGCFDYAQQGGSRYTDPNG					
Consensus	P!NaEqLaQL.GLTianaaeGgATaYannkiagnfdYaqqnnrdt.#pnq					
	101	110	120	130	140	150
	-----+-----+-----+-----+-----					
satA	FLQKDSFKPDOLYILWVGANDYLAYG--ANTEQDAKRYRDAISDAANRMV					
R.sol	IGHNGGAGALTYPYQQQLANFYAASNNTFNGMNDVYFVLGSNDIFFHTT					
Consensus	igqndgagaddlp!qqqgANdYaAsn..fNg##DakrVraainDaanrnt					
	151	160	170	180	190	200
	-----+-----+-----+-----+-----					
satA	LNGAKQILLFNLPLGQNPSSARSQKYVEAVSHVSAYHNKL-LLNLARQLA					
R.sol	AAATSGSGVTPAIATAQVQQAAATDLYGYVKDMIAGKATQVYVFNLPDSSL					
Consensus	aaaakqiglfnaialaQnqqAas#lVgeakdh!aaganql.l1NLarqla					
	201	210	220	230	240	250
	-----+-----+-----+-----+-----					
satA	PTGMVKLFEIDKQFAEMLRDPQNFGLSDVENPCYDGGYVWKPFAATRSYST					
R.sol	TPDGVASGTTGQALLHALYGTFTTLQSGLAGTSARIIDFNAQLTAATIQN					
Consensus	ppdgValgeidqalaealRdpqNfgLqdgeagcsargidfnaqaTaa!qn					
	251	260	270	280	290	300
	-----+-----+-----+-----+-----					
satA	DRQLSAFSPQERLAIAG--NPLLAQAVASPM--ARRSASPLNCEGKMFH					
R.sol	GASFGEFANTSARACDATKINALYPSAGGSSLFCSANTLYASGADQSYLFA					
Consensus	daqlgaanpqaRaadAg..NaLlaqAgaSp\$...Arrlaapgad#gk\$Fa					
	301	310	320	330		
	-----+-----+-----					
satA	DQVHPTTVVHAALSERAAFTIETQYEFLAH					
R.sol	DGVHPTTAGHRLIASNVLARLLA--DNVAH					
Consensus	DqVHPTTagHaaiaraaariae..#nLAH					

Figure 33

Pfam	*->ivafGDSltddggg.....ayygdsdgggwgagladritsla..rlrargrgvdr	
Sriml	38	YVALGDSYSSGVG.....agSYDSSSGSCKRSTKSYPALWAAS.....HTGTRF 81
Scoe1	5	YVAVGDSFTEG---VGDPPDGAFVWADRLAVLL..ADRRPEGDFTY 47
Scoe2	10	LVAVGDSFTEG---MSDLLPDGSYRGWADLLATRM...AARSPGFRY 50
Scoe3	239	VVAFGDSITDG---ARSQSDANHRWTDVLAARLHEAA..GDGRDTPRYSV 283
Scoe4	75	LMMLGDSSTAAG---QGVHRAGQTPGALLASG..LAAVAERPVR 113
Scoe5	66	VAAVGDSITRGFD.....acAVLSDCPEVSWATGSSAKVDSLAvrLLKADAAEHS 116
Ahyd1	28	IVMFGDSLSDTGKmyskmyrgylpssppyYEGRFSGNPVWLEQLTNEFPGLTIANEAEGGPTAVA 91
Asall	28	IVMFGDSLSDTGKmyskmyrgylpssppyYEGRFSGNPVWLEQLTKQF-----PGLTI 79
Ahyd2	40	IVMFGDSLSDTGKmyskmyrgylpssppyYEGRFSGNPVWLEQLTKQFPGLTIANEAEGGATAVA 103
▼		
Pfam	fnrgisGrtsdGrlvvDarlvatllFlaqflGlnlpPYLsgdflrGANFAsagAtIlgtslipflni	
Sriml	82	NFTACSGAR----- 90
Scoe1	48	TNLAVRGRL----- 56
Scoe2	51	ANLAVRGRL----- 59
Scoe3	284	VNEGISGNR----- 292
Scoe4	114	GSVAQPGAC----- 122
Scoe5	117	WNYAVTGAR----- 125
Ahyd1	92	YNKISWNP----- 100
Asall	80	ANEAEAGAT----- 88
Ahyd2	104	YNKISWNP----- 112
▼		
Pfam	QvqFkdfkskvlelrqa.....lgllqellrlvpvdakspdlvtimigtndl...itvakfgpks	
Sriml	91	-----TGDLVLAQQLTPVNSGTDLSITIGGNDagfadTMTTCNLQG 131
Scoe1	57	-----LDQIVAEQVPRVVG LAPDLVSFAAGGNDI.....I----- 86
Scoe2	60	-----IGQIVDEQVDVAAAMGADVITLVGGGLNDT..... 88
Scoe3	293	-----LLTSRPGRPA.....DNPSGLSRFQRDVLERTNVKAVVVVLGVNDV..... 333
Scoe4	123	-----SDDLDRQVALVLAEPDRVPDICVIMVGANDV..... 153
Scoe5	126	-----MADLTAQVTRAAQREPELVAVMAGANDA.....CR 155
Ahyd1	101	-----YQVI.....NNLDYEVTQFLQKDSFKPDDLVLVWVGANDY.....LA 137
Asall	89	-----AVAYNKISWNPkyqvynNLDYEVTQFLQKDSFKPDDLVLVWVGANDY.....LA 137
Ahyd2	113	-----YQVI.....NNLDYEVTQFLQKDSFKPDDLVLVWVGANDY.....LA 149
▼		
PfamtksdrnvsvpefrdnrlrklkrLrsangariiiilitlvllnlpl.....plGCl	
Sriml	132	esaclarIAKARAYIQQLTPAQLDQVYDAIDSRAPAA-----QVVVLGYP----- 176
Scoe1	87	-----RPGTDPDEVAERFELAVAALT-AAAGTVLVTTFGDFTRGV----- 125
Scoe2	89	-----LRPKCDMARVRDILLTQAVERLAPHCEQLVLMRSP----- 122
Scoe3	334	-----LNSPELADRDAITGLRTLVDRAHAGRLRVVGATITPFGGYGG----- 376
Scoe4	154	-----THRMPTATRSVRHLSSAVRRLR-TAGAEVVVGTCPDLTIE----- 192
Scoe5	156	-----STTSAMTPVADFRAQFEEAMATLR-KKLPKAQVYVSSIPDLKRLwsqgrtnplgkQVWKL 214
Ahyd1	138	-----YGWNTQDAKRVRDAISDAANRMV-LNGAK-----EILLFNLP----- 174
Asall	138	-----YGWNTQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP----- 174
Ahyd2	150	-----YGWNTQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP----- 186
▼		
Pfam	pq.klalalassknvdatgclerlneavadynealrelaei.ek.l.q.aqlrkdgldpdlkeanvpy	
Sriml	177	---RFYKLGSCAVGLSEKSRAAINAAADDINAVTAKRA-----ADHGFAF 219
Scoe1	126	---VLKHLRGKIATYNGHVRAIA-----DRYGCVP 152
Scoe2	123	---GROGPVLERFRPRMEALFAVIDDLA-----GRHGAVV 154
Scoe3	377	---YTEARETMREQVNEEIRSGRVFDTVVDFDKALRDPY----- 412
Scoe4	193	---RVQRPLRWLaRraSrQIAAAQTIGAVEQGGRTVSL 227
Scoe5	215	GLcPSMLGDADSLDSAATLRRNTVRDRVADYNEVLREVC---AkDRRCSDDGAVHEFRFGT 273
Ahyd1	175	---DLGQNPSARSQKVVEASHVSAYHNQLLLNLA---ROLAPTGMVKLFEIDKQF 224
Asall	175	---DLGQNPSARSQKVVEASHVSAYHNQLLLNLA---ROLAPTGMVKLFEIDKQF 224
Ahyd2	187	---DLGQNPSARSQKVVEASHVSAYHNQLLLNLA---ROLAPTGMVKLFEIDKQF 236
▼		
Pfam	VDlysifqldldgiqnpsayv.y....GFeet.kaCCGyGgr.yNyn.rv.CGnag.l.ck.vtakaC	
Sriml	220	GDVNT-----TFAGHEICSGAPwL.HS.VT----- 242
Scoe1	153	LDLWSLRSVQDRRA----- 166
Scoe2	155	VDLYGAQSLADPRM----- 168
Scoe3	413	----- 413
Scoe4	228	GDLLGPEFAQNPREL----- 242

Scoe5 274 DQL----- 276
 Ahyd1 225 AEMLRDPQNFGSLSDQRNACYgGsyvwKPFASrSASTDSQLSaFNPQeRLaIAGNP1LaQAvASPMMA 291
 Asa11 225 AEMLRDPQNFGSLSDVENPCYdGgyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNP1LaQAvASPMAR 291
 Ahyd2 237 AEMLRDPQNFGSLSDVENPCYdGgyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNP1LaQAvASPMAR 303

Pfam .dassy11.atl1fwDgf.HpsekGykavAeal<-*
 Srim1 243 .-----LPVENSyHPTANGQSKGYLPV 263
 Scoe1 167 .-----WDADRL.HLSPEGHTRVALRA 186
 Scoe2 169 .-----WDVDRL.HLTAEGHRRVAEAV 188
 Scoe3 413 .-DPRRMRsDYDSGDHL.HPGDKGYARMGAVI 441
 Scoe4 243 .-----FGPDNY.HPSAEGYATAAMAV 262
 Scoe5 277 .-----SHWDWF.HPSVDGQARLAEIA 296
 Ahyd1 292 rSASTLNCeGKMFWQV.HPTTVVHAALSEPA 322
 Asa11 292 rSASPLNCeGKMFWQV.HPTTVVHAALSER 322
 Ahyd2 304 rSASPLNCeGKMFWQV.HPTTVVHAALSER 334

Figure 34

Pfam	*->ivafGDSltdggg.....ayygsdsgggwgagladrltsla..rlrargrgv		
Srim1	38	YVALGDSYSSGVG.....agSYDSSSGSCKRSTKSYPALWAAS.....HTGTRF	81
Scoe1	5	YVAVGDSFTEG--.....--VGDPGPDGAFVGVWADRLAVLL..ADRRPEGDFTY	47
Scoe2	10	LVAVGDSFTEG--.....--MSDLLPDGSYRGWADLLATRM..--AARSPGFRY	50
Ahyd1	28	IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSGNPVWLEQLTNEFPGLTiaNEAEGGPTAVA	91
Asa11	28	IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSGNPVWLEQLTKQF-----PGLTI	79
Ahyd2	40	IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSGNPVWLEQLTKQFPGLTiaNEAEGGATAVA	103
▼			
Pfam	fnrgisGrtsdGr1vvDarlvatlFlaqf1GlnlpPYLsgdflrGANFAsagAtilgtslipflni		
Srim1	82	NFTACSGAR-----	90
Scoe1	48	TNLAVRGRL-----	56
Scoe2	51	ANLAVRGKL-----	59
Ahyd1	92	YNKISWNPK-----	100
Asa11	80	ANEAEAGGAT-----	88
Ahyd2	104	YNKISWNPK-----	112
▼			
Pfam	QvqFkdfkskvlelrqa.....lgllgellrlvpvldakspdlvtimiGtND1...itvakfgpks		
Srim1	91	-----TGDVIAKQLTPVNSGTDLVSTIGGNDagfaDTMTTCNLQG	131
Scoe1	57	-----LDQIVAEQVPRVVLAPDLVSFAAGGNDI.....I-----	86
Scoe2	60	-----IGQIVDEQVDVAAAMGADVITLVGGGLNDT.....	88
Ahyd1	101	-----YQVI.....NNLDYEVTVQFLQKDSFKPDDLVLVWVGANDY.....LA	137
Asa11	89	-----AVAYNKISWNpkyqvyNNLDYEVTVQFLQKDSFKPDDLVLVWVGANDY.....LA	137
Ahyd2	113	-----YQVI.....NNLDYEVTVQFLQKDSFKPDDLVLVWVGANDY.....LA	149
▼			
PfamtksdnrvsvpefrdnlrkklkrLrsangariililitlvi1nlp1GCl		
Srim1	132	esaclarIAKARAYIQQTLPALQDQVYDAIDSRAFAA-----QVVVLGYP-----	176
Scoe1	87	-----RPGTDPDEVAERFELAVAALT-AAAGTVLVTTFGDFTRGVP-----	125
Scoe2	89	-----LRPKCDMARVRDLTQAVERLAPHCEQLVLMRSP-----	122
Ahyd1	138YGWNTQDAKRVRDAISDAANRMV-LNGAK-----EILLFNLP-----	174
Asa11	138YGWNTQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP-----	174
Ahyd2	150YGWNTQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP-----	186
▼			
Pfam	pqklalalassknvdatgclerlneavadynealrelaeieklqaqlrkdgldplkeanvpy		
Srim1	177	--RFYKLGGSACVGLSEKSRAAINAAADDINAVTAKRA-----ADHGFAF	219
Scoe1	126	-----VLKHLRGKIATYNGHVRAIA-----DRYGCPV	152
Scoe2	123	-----GRQGPVLERFRPRMEALFAVIDDLA-----GRHGAVV	154
Ahyd1	175	-----DLGQNPSARSQKVVEASHVSAHYHNQLLLNLA-----RQLAPTGMVKLFEDKQF	224
Asa11	175	-----DLGQNPSARSQKVVEASHVSAHYHNQLLLNLA-----RQLAPTGMVKLFEDKQF	224
Ahyd2	187	-----DLGQNPSARSQKVVEASHVSAHYHNQLLLNLA-----RQLAPTGMVKLFEDKQF	236
▼			
Pfam	VDlysifqldgiqnpsayv.y...GFeet.kaCCGyGgr.yNyn.rv.CGnag.l.ck.vtakaC		
Srim1	220	GDVNT-----TFAGHEICSGAPwL.HS.VT-----	242
Scoe1	153	LDLWSLRSVQDRRA-----	166
Scoe2	155	VDLYGAQSLADPRM-----	168
Ahyd1	225	AEMLRDPQNFGLSQDNACyGgyvwKPFASrSASTDQLSaFNPQeRLaIAGNP1LaQAvASPMAR	291
Asa11	225	AEMLRDPQNFGLSQDNACyGgyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNP1LaQAvASPMAR	291
Ahyd2	237	AEMLRDPQNFGLSQDNACyGgyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNP1LaQAvASPMAR	303
▼			
Pfam	.dassyll.atlfdwDgf.HpsekGykavAeal<-*		
Srim1	243	-----LPVENSyHPTANGQSKGYLPV	263
Scoe1	167	-----WDADRL.HLSPEGHTRVALRA	186
Scoe2	169	-----WDVDRL.HLTAEGHRRVAEAV	188
Ahyd1	292	rSASTLNCeGKMFWQV.HPTTVVHAALSEPA	322
Asa11	292	rSASPLNCeGKMFWQV.HPTTVVHAALSER	322
Ahyd2	304	rSASPLNCeGKMFWQV.HPTTVVHAALSER	334

Figure 35

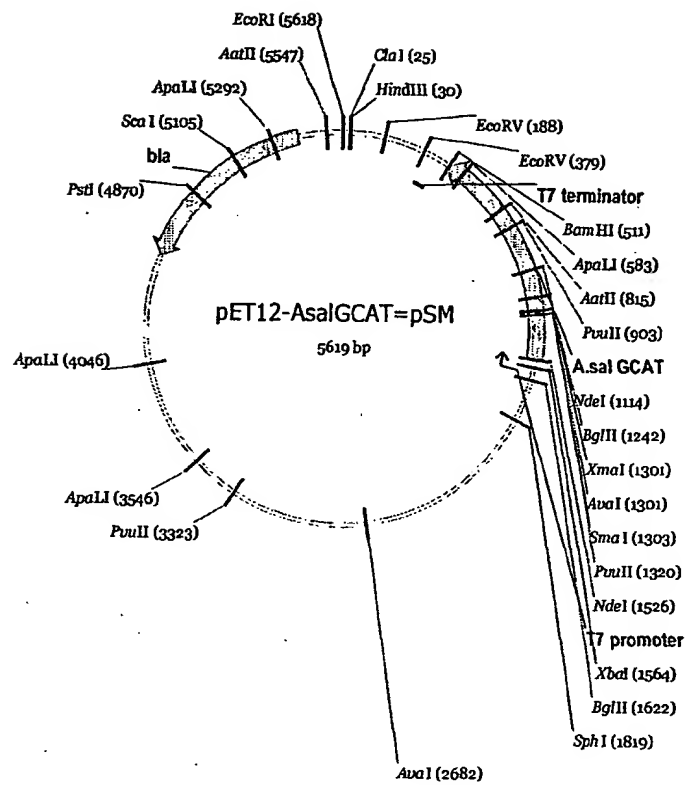


Figure 36



Figure 37

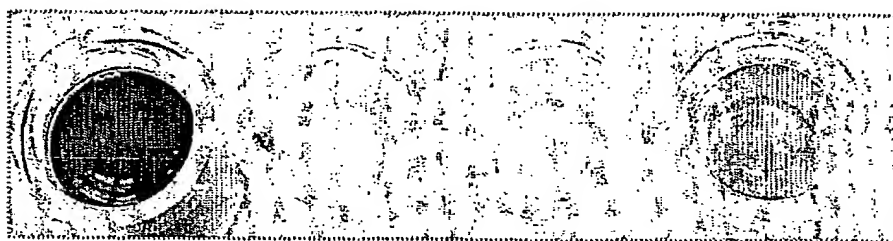


Figure 38

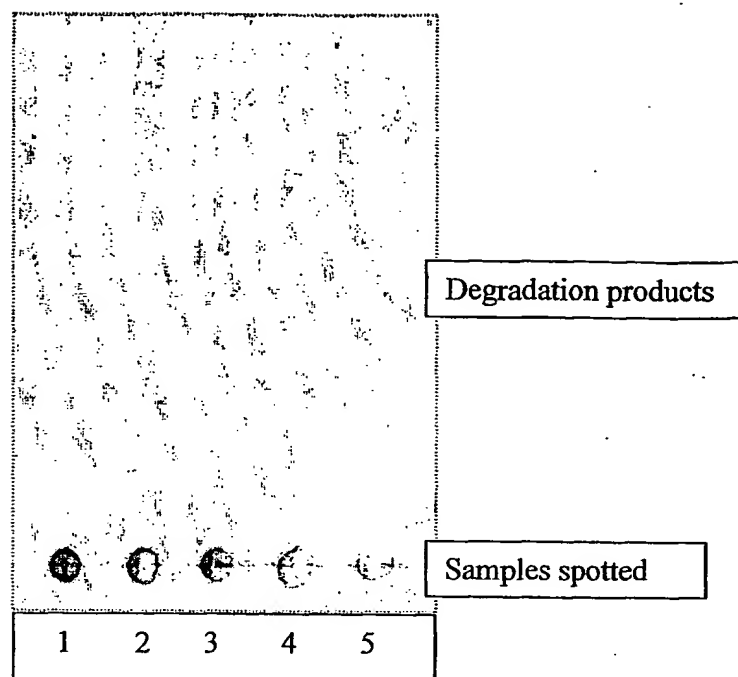


Figure 39

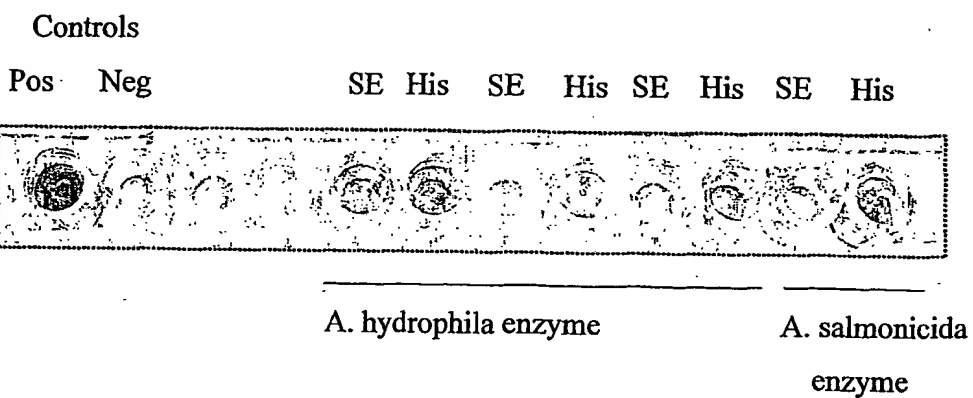


Figure 40.

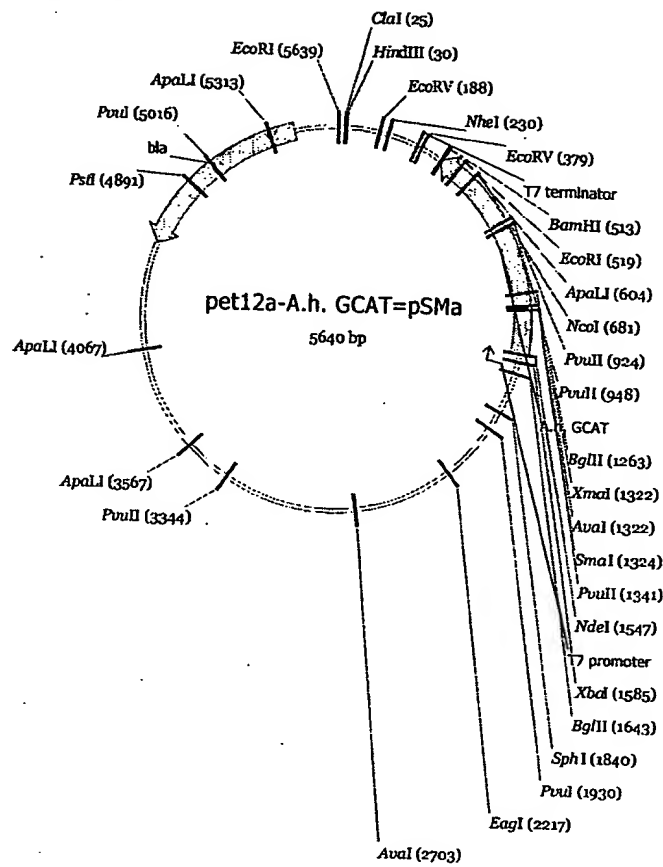


Figure 41:

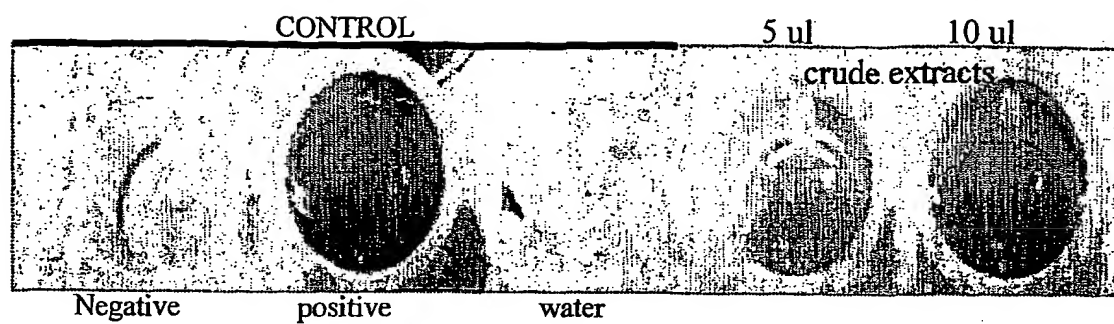


Figure 42

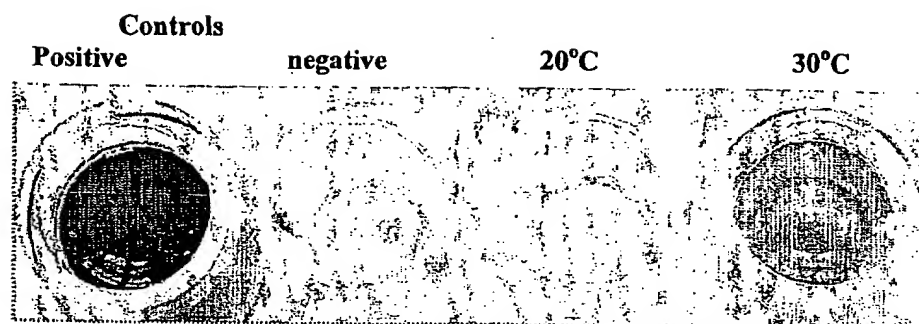


Figure 43

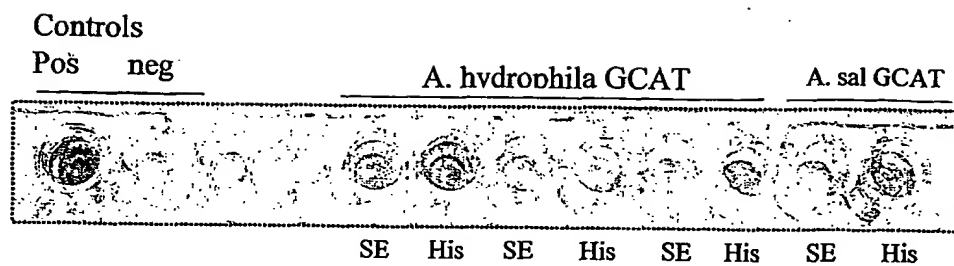


Figure 44

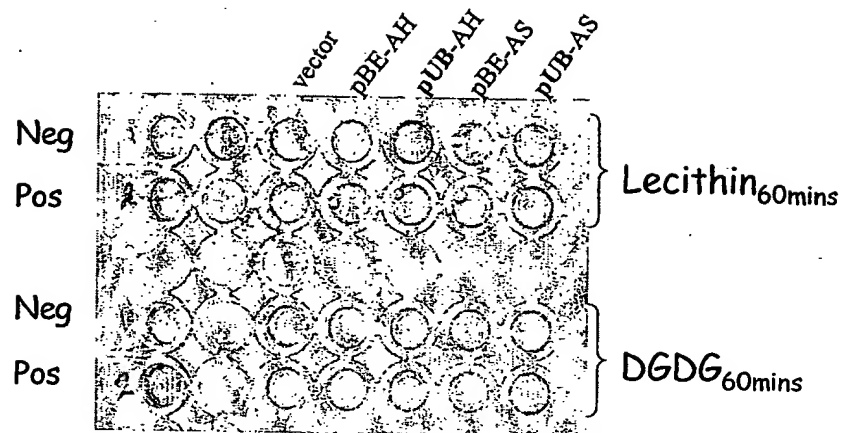


Figure 45

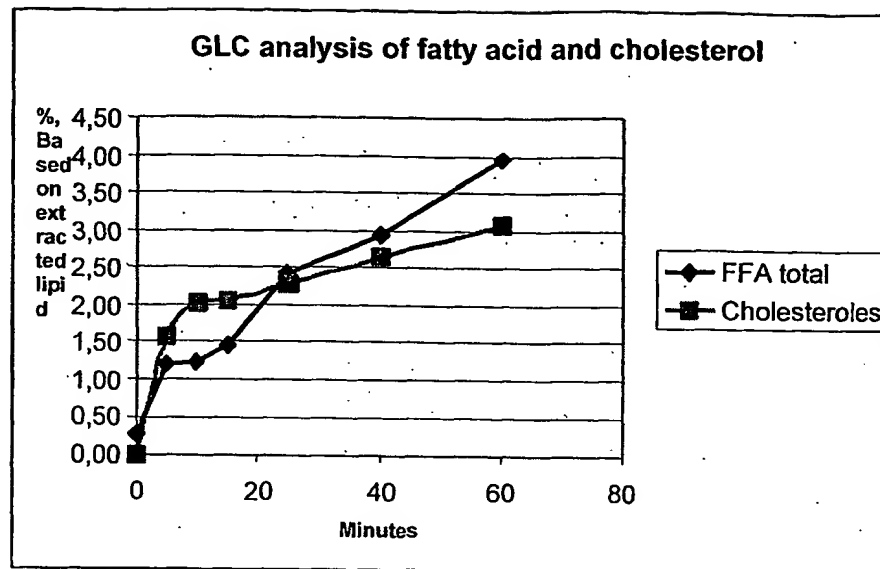


Figure 46

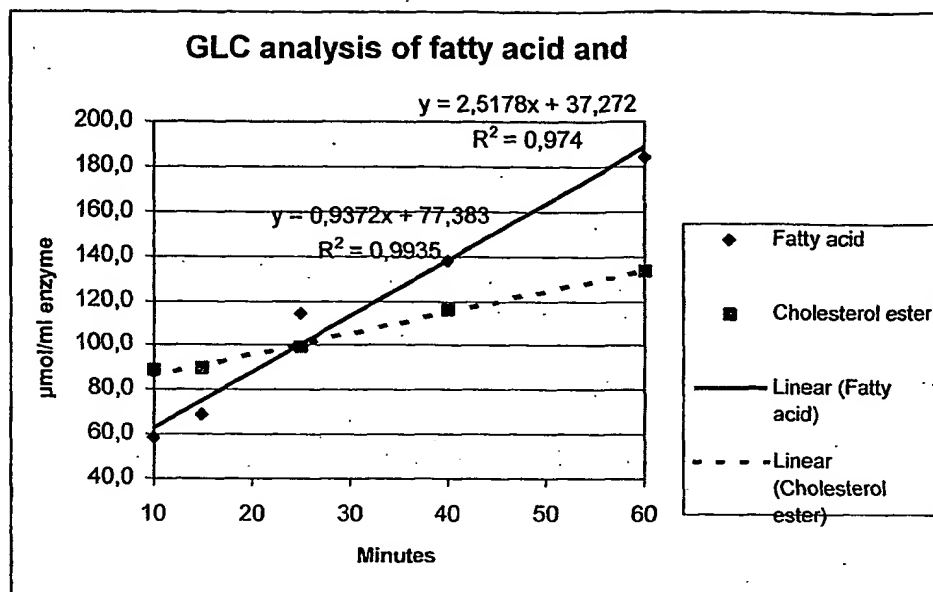


Figure 47

(SEQ ID No. 36)

```
. 1  MEKFKKNFLV GLSAALMSIS LFSATASAAS ADSRPAFSRI VMFGDSLSDT
51  GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTKQFPGLT IANEAEGGAT
101 AVAYNKISWN PKYQVINNLD YEVTQFLQKD SFKPDDLVL VVGANDYLAY
151 GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLENLPDLGQ NPSARSQKVV
201 EAVSHVSAYH NQLLLNLARQ LAPTMGVKLF EIDKQFAEML RDPQNFGLSD
251 VENPCYDGGY VWKPFATRSV STDRLSAFS PQERLAIAGN PLLAQAVASP
301 MARRSASPLN CEGKMFWDQV HPTTVVHAAL SERAATFIAN QYEFLAH**
```

Figure 48 (SEQ ID No. 54)

1 ATGTTTAAAGT TTAAAAAGAA TTTCTTAGTT GGATTATCGG CAGCTTTAAT
TACAAATTCA AATTTTCTT AAAGAATCAA CCTAATAGCC GTCGAAATTA

51 GAGTATTAGC TTGTTTTCGG CAACCGCCTC TGCAGCTAGC GCCGACAGCC
CTCATAATCG AACAAAAGCC GTTGGCGGAG ACCTCGATCG CGGCTGTCTG

101 GTCCCGCCTT TTCCCGGATC GTGATGTTTCG GCGACAGCCT CTCCGATACC
CAGGCGCGAA AAGGGCCTAG CACTACAAGC CGCTGTCTGGA GAGGCTATGG

151 GGCAAAATGT ACAGCAAGAT GCGCGGTTAC CTCCCTCCA GCCCGCCCTA
CCGTTTTACA TGTCGTTCTA CGCGCAATG GAGGGGAGGT CGGCGGGAT

201 CTATGAGGCG CGTTTCTCCA ACGGACCCGT CTGGCTGGAG CAGCTGACCA
GATACTCCCG GCAAAGAGGT TGCCTGGGCA GACCGACCTC GTCGACTGGT

251 AACAGTTCCC GGGTCTGACC ATCGCCAACG AAGCGGAAGG CGGTGCCACT
TTGTCAAGGG CCCAGACTGG TAGCGGTTGC TTCGCCTTCC GCCACGGTGA

301 GCCGTGGCTT ACAACAAGAT CTCCTGGAAT CCAAGTATC AGGTATCAAA
CGGCACCGAA TGTTGTTCTA GAGGACCTTA GGGTTCATAG TCCAGTAGTT

351 CAACCTGGAC TACGAGGTCA CCCAGTTCTT GCAGAAAGAC AGCTTCAAGC
GTTGACCTG ATGCTCCAGT GGGTCAAGAA CGTCTTCTG TCGAAGTTCG

401 CGGACGATCT GGTGATCCTC TGGGTCGGTG CCAATGACTA TCTGGCCTAT
GCCTGCTAGA CCACTAGGAG ACCCAGCCAC GGTACTGAT AGACCGGATA

451 GGCTGGAACA CGGAGCAGGA TGCCAAGCGG GTTCGCGATG CCATCAGCGA
CCGACCTTGT GCCTCGTCCT ACGGTTCCGC CAAGCGCTAC GGTAGTCGCT

501 TGC GGCCAAC CGCATGGTAC TGAACGGTGC CAAGCAGATA CTGCTGTTCA
ACGCCGGTTG GCGTACCATG ACTTGCCACG GTTCGTCTAT GACGACAAGT

551 ACCTGCCGGA TCTGGGCCAG AACCCGTCAG CTCGCAGTCA GAAGGTGGTC
TGGACGGCTT AGACCCGGTC TTGGGCAGTC GAGCGTCAGT CTTCCACCAG

601 GAGGCGGTCA GCCATGTCTC CGCCTATCAC AACCAGCTGC TGCTGAACCT
CTCCGCCAGT CGGTACAGAG GCGGATAGTG TTGGTCGACG ACGACTTGGA

651 GGCACGCCAG CTGGCCCCCA CCGGCATGGT AAAGCTGTTT GAGATCGACA
CCGTGCGGTC GACCGGGGGT GGCGGTACCA TTTCGACAAG CTCTAGCTGT

701 AGCAATTGTC CGAGATGCTG CGTGATCCGC AGAACTTCGG CCTGAGCGAC
TCGTAAACG GCTCTACGAC GCACTAGGCG TCTGAAGCC GGAATCGCTG

751 GTCGAGAACC CCTGCTACGA CGGCGGCTAT GTGTGGAAGC CGTTGCCAC
CAGCTCTTGG GGACGATGCT GCCGCCGATA CACACCTTCG GCAAACGGTG

801 CCGCAGCGTC AGCACCAGAC GCCAGCTCTC CGCCTTCAGT CCGCAGGAAC
GGCGTCGAG TCCTGGCTGG CGGTCGAGAG GCGGAAGTCA GCGTCCTTG

851 GCCTCGCCAT CGCCGGCAAC CCGTGTCTGG CACAGGCCGT TGCCAGTCTT
CGGAGCGGTA GCGGCCGTTG GCGACGACC GTGTCCGCA ACGGTCAGGA

901 ATGGCCCGCC GCAGCGCCAG CCCCTCAAC TGTGAGGGCA AGATGTTCTG
TACCGGGCGG CGTCGCGGTC GGGGGAGTTG AACTCCCGT TCTACAAGAC

951 GGATCAGGTA CACCCGACCA CTGTCGTGCA CGCAGCCCTG AGCGAGCGCG
CTAGTCCAT GTGGGCTGGT GACAGCACGT GCGTCGGGAC TCGTCGCGC

1001 CCGCCACCTT CATCGGAAC CAGTACGAGT TCCTCGCCCA CTGATGA
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Figure 49

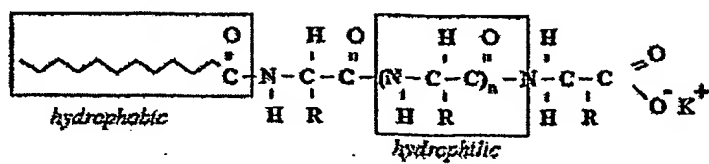


Figure 50

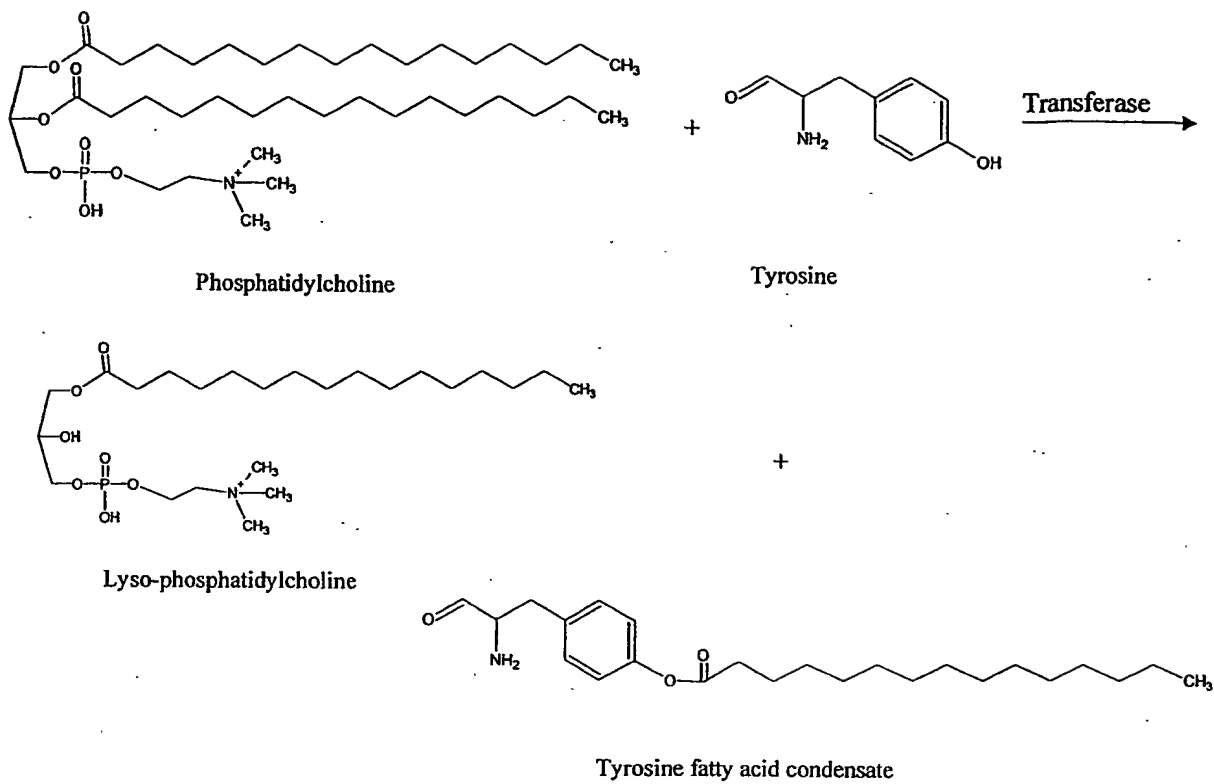
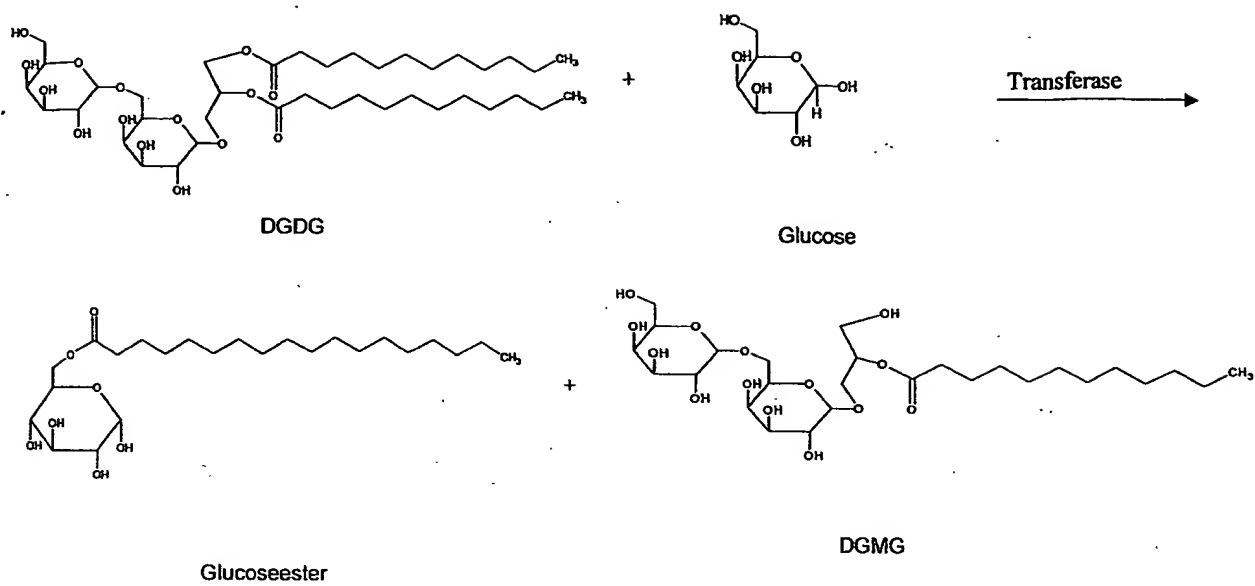


Figure 51



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